REVIEWS IN Aquaculture

Reviews in Aquaculture (2013) **5** (Suppl. 1), S1–S25 doi: 10.1111/j.1753-5131.2012.01082.x

Microbiology and immunology of fish larvae

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Received 27 December 2011; accepted 13 June 2012.

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Abstract

For most marine aquaculture species, one of the main bottlenecks is the stable production of high quality juveniles. The high and unpredictable mortality in the first weeks after hatching of marine fish larvae remains a challenging problem that needs to be solved. The severity of the problem differs between species, but cannot be considered adequately solved for any species. Both scientific evidence and experience in hatcheries for a variety of fish, shrimp and shellfish species are accumulating as support for the hypothesis that detrimental fish–microbe interactions are the cause of these problems. Host–microbe interactions in reared fish are still poorly understood, except for a few pathogens, and empirical data of the quality required to test this hypothesis, are lacking. This article provides an overview on the current knowledge of the microbial environment of fish larvae, including methodological aspects to characterize the microbial community (both using culture-dependent and culture-independent methods). Further, the current knowledge of the immunology of fish larvae is reviewed, including recent advances in the understanding of toll-like receptors, inflammatory cytokines, mast cells and piscidins, and the ontogeny of the adaptive immune system. Finally, we provide an overview of the state of the art with respect to steering of microbial communities associated with fish larvae – both steering of community composition and of its activity (e.g. by quorum sensing interference).

Key words: host–microbe interaction, mast cell, microbial ecology, piscidin, quorum sensing.

Introduction

For a lot of aquatic species, the cost effective commercial production of larvae is still a bottleneck. In general, symptoms associated with bottlenecks can be observed, whereas the causal relationships may be 'hidden'. In aquaculture, bottlenecks exist at all life stages, i.e. throughout the production cycle. However, for most marine aquaculture species one of the main bottlenecks is the stable production of high quality juveniles. The main symptoms observed are poor growth of individuals, a sudden decrease in survival, malformation, and a lack of reproducibility both between independent trials and replicates within the same trial, even with full sibling groups (Støttrup 1993; Vadstein *et al.* 2004). Some of the problems associated with juvenile quality are visible only in later stages (Logue *et al.* 2000). This emphasizes how important the first feeding stage is for the rearing process in total.

The raising of marine fish larvae has only become feasible on a commercial scale because of major advances in the knowledge of nutritional requirements (e.g. Coutteau *et al.* 1997; Merchie *et al.* 1997; Sorgeloos *et al.* 2001). However, the high and unpredictable mortality (sometimes more

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than 80%) in the first weeks after hatching remains a challenging problem that needs to be solved. The severity of the problem differs between species, but cannot be considered adequately solved for any species.

Different factors have been proposed to cause the observed symptoms, including the quality of gametes, inadequate nutrition, suboptimal physicochemical conditions and detrimental fish–microbe interactions (Vadstein *et al.* 1993). Traditionally, the focus has been on the first three factors. Considerable progress has been achieved related to these factors during the past decades, and for some species, such as sea bass, this progress has made the industry viable. However, performance still varies considerably for identical treatments, even between replicates of the same sibling group. The fact that some rearing units perform well indicates that egg quality, nutrition and physiochemical conditions which are fairly well controlled and kept identical, are adequate. As these conditions are kept constant, they cannot explain the variability between replicates and thus, one may hypothesize that detrimental fish–microbe interaction is the only proposed factor that can explain the lack of reproducibility between replicate treatments with full sibling groups. Both scientific evidence (cf. Vadstein *et al.* 2004) and experience in hatcheries for a variety of fish, shrimp and shellfish species are accumulating supporting this hypothesis. For instance, bacteria play an important role in the early survival of turbot larvae, as demonstrated by the relatively high turbot survival rates in the absence of culturable bacteria (55% after 14 days feeding on axenic rotifers) (Munro *et al.* 1995). Similar results were obtained in the study of Tinh *et al.* (2008), where a high survival (with low variability) of turbot larvae was observed in the first week due to the addition of the antibiotic rifampicin. Host–microbe interactions in reared fish are still poorly understood, except for a few pathogens, and empirical data of the quality required to test this hypothesis are lacking (Vadstein *et al.* 2004). This article aims at reviewing the state of the art on microbiological interference in larviculture, focusing both on the microbial and immunological aspects.

The microbial environment of fish larvae

When compared with land-based animals, aquatic animals live in a 'hostile' environment with a high bacterial load (Verschuere *et al.* 2000). Typically, 10^6 bacteria and 10^8 viruses are found in 1 mL of seawater, but densities are normally considerably higher in environments with higher input of organic matter (e.g. aquaculture systems). The situation is aggravated due to the fact that fish, feed and excretion products are present in the same matrix, namely the rearing water. Hence, this environment is conducive to the proliferation of microorganisms (beneficial, neutral or pathogenic). In the past, the focus has been on pathogenic bacteria and their role in disease. However, although detrimental effects have been found to be related to the bacterial biota during first feeding of fish, few pathogens have been identified (Vadstein *et al.* 1993; Skjermo & Vadstein 1999; Sandlund & Bergh 2008). This is in contrast to the on-growing stage, where most of the problems can be related to specific pathogens.

Generally, microbes can be transferred or interact with the larvae in a number of ways (Fig. 1). The bacterial sources can be divided in two main categories, external and internal. To what extent external sources influence the microbial conditions in the rearing environment depends on the openness of the system. For indoor first feeding tank systems, microbes coming from the live feed cultures, including algae, are the main external input of microbes. Traditionally there has been more focus on microbes in intake water, and water treatment to reduce the density of bacteria is more or less a standard procedure. Control measures directed towards microbes associated with the live feed (zooplankton and algae) has, on the other hand, mostly been neglected. This is in spite of the fact that detrimental effects due to bacteria associated with live feed are well known (Perez-Benavente & Gatesoupe 1988; Olsen *et al.* 2000).

The outer surface of the fish is colonized by bacteria in the water, whereas the intestine is generally affected by bacteria entering via active uptake from the water (Reitan *et al.* 1998) and by bacteria associated with the feed. The number of microbes associated with live feed may exceed 10^{10} cells g^{-1} (Skjermo & Vadstein 1993). However, there is also a significant enrichment of microbes inside the rearing tanks, which is mainly based on organic material

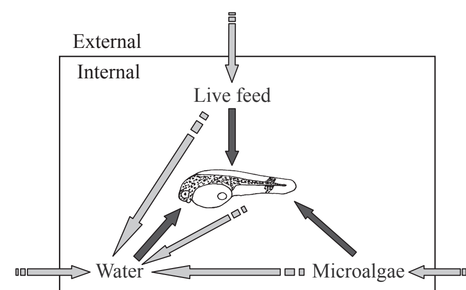

Figure 1 Important microbial sources interacting with mucosal surfaces of larval fish. Various external sources of microbes (blue/grey arrows) such as water, live feed and microalgae, enter the rearing environment and interact with the fish (red/black arrows). Internally, the rearing environment is enriched by microbes due to defaecation by fish or live feed, or indirectly through growth based on organic matter released by defaecation by animals or exudation by microalgae. After Vadstein *et al.* (2004).

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released in the tanks. Particularly the defaecation processes of both fish and live feed may constitute a significant input to the water of both microbes and substrates for microbial growth. Bacterial growth in first feeding tanks may be fairly high, with growth rates in the range of 0.5–2 divisions per day (Vadstein *et al.* unpublished). The dynamics of the microbial population in the tanks is also evident from the fact that the bacterial biota of live feed changes after transfer to the tanks. This includes both a reduction in the numbers and a change in the composition of the bacterial biota (Øie *et al.* 1994; Olsen *et al.* 2000). One may hypothesize that live feed represents the heaviest load of bacteria to the larvae, except for the first days after opening of the mouth when active uptake directly from the water is significant (Reitan *et al.* 1998). It is important to be aware that the direct, active uptake is 2–3 orders of magnitude higher than the drinking rate (Reitan *et al.* 1998), which may be explained by the DLVO theory (Hermansson 1999).

It appears that the microbiota of newly hatched larvae is largely established in a non-selective manner, even though host preference and competitive ability also seem to be of importance (Makridis *et al.* 2000a; Verschuere *et al.* 2000). Most previous studies on characterization of the microbiota have pooled individuals to one composite sample and used culture dependent approaches. A complication of the general picture is indicated by a study on approximately 18 day-old Atlantic cod larvae, where it was found that the individual variation in the composition of the microbiota within one tank was as comparable to the variation between rearing facilities with large differences in cultivation regime (Fjellheim *et al.* 2012). Similar observations have been made with various mammals (Vanhoutte *et al.* 2004; Eckburg *et al.* 2008). Moreover, for the cod larvae a low correlation was found between a culture-dependent and a culture-independent approach, in terms of both quantity and diversity of microbes. It still needs to be verified whether this large individual variation also exists in other species and developmental stages, and more importantly, to what extent this variation has biological implications for the host.

The discussion thus far has mainly considered quantitative aspects. However, the identity of the microbes that are present and dominating is probably more important than the actual numbers. Currently applied intensive production methods tend to increase the carrying capacity for microbes of aquaculture systems (increase in bacterial load) and to select for opportunistic microbes. The reasons for such an unfavourable development are the presence of a high load of organic matter, with large oscillations in this load in time and space, and direct perturbations of the microbial community. Critical factors that sustain such a negative development are the decimation of bacteria in in-flowing water without controlled re-colonization of the microbial community, the addition of high loads of bacteria and organic matter to the system together with the feed, and the presence of high levels of organic matter as faeces from live feed and larvae or as dead larvae (Vadstein *et al.* 2004). According to the r/K-concept (cf. Andrews & Harris 1986), such oscillating conditions select for r-strategists; i.e. opportunistic species that can multiply rapidly. Pathogens are often characterized as r-strategists (Andrews 1984), together with a large number of non-pathogenic, opportunistic species that may cause some of the problems experienced in the rearing of marine larvae.

Apart from practical problems, the stochastic colonization of larvae by micro-organisms also creates experimental problems as it is very hard to establish reproducible experimental conditions. Basically, under normal experimental conditions, the microbial community of larvae used in experiments repeated in time cannot be considered identical in composition and/or in activity. Hence, from a microbiological point of view, repeating experiments is almost impossible. This problem can be solved by starting with axenic larvae and subsequently adding known micro-organisms. However, this also creates limitations as only characterized and culturable micro-organisms can be tested in this way. Such experimental gnotobiotic conditions have been used in the past (for a review, see Marques *et al.* 2006a), and have been developed recently for larvae of European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) (Marques *et al.* 2006b; Dierckens *et al.* 2009; Forberg *et al.* 2011a,b). It can be expected that such experimental approaches will be instrumental in further unravelling host–microbial interactions in the early larval stages.

Methodological aspects of microbial community characterization

Prior to the advent of culture-independent molecular biological tools, the characterization of the microbiota associated with fish eggs and larvae was dependent on the more 'conventional' microbiological cultivation techniques. In a pioneering paper, Oppenheimer (1955) demonstrated the presence of bacteria on the surface of live cod (*Gadus morhua*) eggs, and suggested that the bacteria accumulated on the eggs until present in sufficient numbers to be injurious. This phenomenon had already been partly described by Dannevig (1919) who noticed that cod eggs could become overgrown with filamentous organisms, which according to his description probably were *Leucothrix mucor*. The genera *Pseudomonas, Aeromonas* and *Flavobacterium* were found by Hansen and Olafsen (1989) to be dominant on cod and halibut

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(*Hippoglossus hippoglossus*) eggs. They also found members of the genera *Moraxella* and *Alcaligenes* on halibut eggs, but in contrast to cod eggs, halibut eggs showed only sparse presence of *L. mucor*. The composition of the bacterial microbiota adhering to eggs was variable among egg batches of halibut (Hansen *et al.* 1992; Bergh 1995).

Studies of the intestinal microbiota of larval and adult fish were questioned in the 1960s and 1970s, but later studies demonstrated the presence of a specialized microbiota in fish (reviewed by Cahill 1990). A range of culture-dependent studies was performed describing the taxonomic composition of fish intestinal microbiota and its roles (reviewed by Hansen and Olafsen (1999) and Ringø and Birkbeck (1999)). A two-step pattern of colonization has been demonstrated in fish that possess a distinct yolk sac stage and where no exogenous feeding occurs (Ringø *et al.* 1995). In halibut, the introduction of the second level of colonization was characterized by a shift from a non-fermentative microbiota dominated by the *Cytohaga/Flexibacter/Flavobacterium* group to a fermentative microbiota dominated by the *Vibrio/Aeromonas* group) (Bergh *et al.* 1994; Bergh 1995).

Today, the utilization of culture-dependent methods is partly replaced by molecular methods such as denaturing gradient gel electrophoresis of PCR amplified 16S rDNA (PCR/DGGE). However, for specific purposes, such as the search for probiotic candidates (Hjelm *et al.* 2004) or disinfectants (Salvesen & Vadstein 1995), culture-dependent methods provide advantages and are still used. After all, it is still often necessary to be able to study the organisms of interest in culture.

One of the most popular fingerprinting techniques to characterize the composition and diversity of a microbial community is the PCR/DGGE method (Muyzer *et al.* 1996). DGGE enables us rapidly to screen multiple samples and to obtain valuable information about community changes and differences, distinguishing between communities and identifying community members. For instance, comparisons between groups with high mortality and low mortality will be facilitated, as PCR/DGGE enables a rapid overview of the presence or absence of certain bands, representing microbial species or subspecies. DNA from the bands can be sequenced and used for identification. Important bands can be used to design specific primers or probes, which in turn can be used in PCR protocols for the detection and quantification of certain microorganisms or for fluorescent *in situ* hybridization to determine the location of these species on the fish. Studies from a hatchery for Great Scallop (*Pecten maximus*) larvae revealed that suspected opportunistic pathogens were present in larval cultures experiencing high mortality (Sandaa *et al.* 2003). The PCR/DGGE technique has proven to be an efficient tool for monitoring the presence and the proliferation of opportunistic pathogens in aquaculture systems (Sandaa *et al.* 2008). PCR/DGGE has also been used to describe the intestinal microbiota of halibut larvae from different geographical origins and different ontogenetic stages (Jensen *et al.* 2004), and of the microbiology of developing cod larvae (Brunvold *et al.* 2007; McIntosh *et al.* 2008).

Microbial ecologists have definitely shifted from the use of culture-dependent techniques to culture-independent techniques. However, a sound interpretation of the results of culture-independent techniques is as important as for culture-dependent techniques. Quantitative errors might be introduced both in nucleic acid extraction and PCR; bacterial cells in nature exhibit different degrees of resistance to cell lysis, which is necessary for nucleic acid extraction, and PCR is a technique for which biases have been shown to exist (Wintzingerode *et al.* 1997). This makes PCR/DGGE unsuitable for quantitative measurements and extraction protocols need to be evaluated.

Bands at the same position in the gel have the same melting behaviour, but not necessarily the same sequence (Muyzer *et al.* 1993). PCR amplification of 16S rDNA also involves the problem that some species have multiple, heterogenous copies of 16S rDNA on the bacterial genome. This may result in heteroduplex formation and possibly multiple bands of amplified 16S rDNA fragments from one species. In the DGGE profiles of bacterial isolates sampled at a Norwegian cod hatchery, all isolates displayed multiple bands (Brunvold *et al.* 2007). Similar results were obtained by Dahlløf *et al.* (2000), where eight of 14 bacterial isolates displayed multiple bands for 16S rDNA when analysed by PCR/DGGE.

Other genes than 16S rDNA should be included in the DGGE work Dahlløf *et al.* (2000). Santos and Ochman (2004) evaluated 10 genes spanning an array of genomic locations and functional categories, and also developed primer sets that targeted each gene, including *rpoB*, *recA* and *gyrB.* The gene for the RNA polymerase beta subunit (*rpoB*) has been used in DGGE studies of various environmental bacteria (Peixoto *et al.* 2002; Da Mota *et al.* 2004; Bourne & Munn 2005). *rpoB*-based PCR and DGGE were used by Reid *et al.* (2009) to characterize *Vibrio* species in developing cod larvae, arguing that *rpoB* is a more variable gene than 16S rDNA, allowing to discriminate between species of vibrios. DGGE profiles based on *rpoB* amplification of bacterial isolates displayed single bands. However, the use of *rpoB* may present a taxonomic disadvantage: the database of the sequence is less documented than that of the 16S rRNA gene and consequently DGGE bands cannot easily be attributed to a genus or species (Renouf *et al.* 2006). On the other hand, Peixoto *et al.* (2002) reported that the *rpoB* DGGE profiles comprised fewer bands than the 16S rDNA profiles and were easier

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to delineate and therefore to analyse. Comparison of the community profiles revealed that 16S rDNA-based and *rpoB*-based DGGE were complementary. The use of *recA*, involved in recombination as an alternative phylogenetic marker in the family Vibrionaceae, was evaluated by Thompson *et al.* (2004). The database of this gene is rapidly increasing, facilitating PCR-DGGE targeted identification of bacteria within the Vibrionaceae.

Another drawback of these fingerprinting techniques is that only the most dominant species are examined and that the less dominant members of the microbial community can be missed. These methods generally allow the detection of a phylogenetic group or a bacterial species only if it represents at least 1% of the total microbiota (Seksik *et al.* 2003). Studies of the human intestinal microbiota show that many of the species comprising smaller populations in the intestine, including *E. coli* and *Staphylococcus aureus*, are known to play a significant role in health (Wislinghoff *et al.* 2004). In order to detect less abundant populations, specific fingerprints for relevant microbial groups can be obtained, for example for *Clostridium coccoides-Eubacterium rectale* group, *Lactobacilli, Bifidobacteria, Bacteroides, Veillonella* and *Enterococci* in the case of human intestinal microbiota (Van de Wiele *et al.* 2004). In addition to this, other, more advanced techniques with a higher resolution, such as the combination of phylogenetic and functional gene arrays (PhyloChip and GeoChip), can allow a more in-depth analysis of the microbial community, both based on 16S rDNA and on functional genes. Recent high-throughput sequencing platforms such as 454 FLX pyrosequencing, will allow for sequence-driven metagenomic approaches, and could help to characterize the entire microbiota of an individual and/or a culture system.

One of the main problems using molecular techniques is the interpretation of the data and their link with ecosystem functionality. A setting-independent theoretical interpretation of these fingerprint data has recently been proposed, based on a straightforward processing on three levels of analysis: (i) the richness (Rr) reflecting the carrying capacity of the system; (ii) the dynamics of change (Dy) reflecting the specific rate of species coming to significance; and (iii) community organization (Co), describing the evenness of the microbial community by Lorenz curves (Marzorati *et al.* 2008).

The richness parameter Rr is an estimation of 'who is there' and informs on the carrying capacity of an environment, i.e. whether an environment is very habitable or adverse/exclusive (Verstraete *et al.* 2007). The Rr value can be determined by counting the different bands or peaks in fingerprinting data, by defining the unique Operational Taxonomic Units (OTU), by sequencing or by the number of spots on phylogenetic microarrays. Bell *et al.* (2005) stated that rich microbial communities contribute to high ecosystem functioning because overall more resources are used. Losure *et al.* (2007) described this as the fact that increased richness leads to increased niche complementarity. More different species imply a more efficient use of resources. The opportunities for an invader to find a niche in a highly diverse ecosystem is thus considerably decreased. Alternatively, Bell *et al.* (2005) related ecosystem functioning to high microbial richness because it is more likely that species are present that have a large effect on the functioning. In rich communities it is more likely that at least one of the community members has an antagonistic activity against pathogenic invaders. The beneficial effect of a higher richness on pathogen invasion resistance was described by Roberts *et al.* (2004). It seemed that a lower diversity in the microbial rhizosphere community in dwarf wheat led to a higher susceptibility to invasion by *Pseudomonas*.

The community organization (Co) is an estimation of the evenness of the microbial community and relates to the relative abundances of the different species (Read *et al.* 2011). It can be hypothesized that a balanced Co will contribute to the well-being of the larvae as it is more difficult for a pathogen to invade a community with several equal players relative to a situation with a few dominant species (Ley *et al.* 2006). This corresponds to the observations of van Elsas *et al.* (2002), who found that a more uneven distribution of bacteria in soils resulting from agricultural practices decreased the capacity of the soils to deal with the invasion of pathogens. Wittebolle *et al.* (2009) also mentioned that species evenness can be an important element in managing invasions. Unfortunately, as far as known, no information is available concerning the effect of microbial evenness on the resistance against infections in the intestinal environment of aquatic larvae.

The community dynamics value Dy is an estimation of the rate of change of a microbial community and informs on the number of species that on average come to significant dominance in a given habitat, during a defined time interval (Marzorati *et al.* 2008). Stable communities (low Dy values) represent small reservoirs that limit the influx of new propagules and they might be useful in terms of technical performance, but dangerous in terms of overall adaptability (Verstraete *et al.* 2007). Rr, Do and Dy values that are beneficial to fish larvae are not established yet, as too little systematic data are available on their microbial communities. Hence in the future, processing fingerprinting data as described above will need to prove its value.

Pathogens and challenge models

A considerable number of studies have been published testing the virulence of various pathogens to marine fish

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larvae of different species. Testing of virulence through challenge experiments provides useful information on a given bacterium, for instance on whether it should be considered a primary pathogen (i.e. the cause of the disease) or a secondary invader (i.e. opportunists unable to be the primary cause of the investigated condition). Anecdotal information about bacteria being isolated from diseased marine larvae has limited value as evidence for virulence. A screening of a wide range of bacterial strains isolated from cod for virulence towards yolk sac larvae of cod revealed that, apart from *Vibrio anguillarum,* no strains could cause significant mortality (Sandlund & Bergh 2008).The authors concluded that anecdotal information about bacteria being isolated from diseased marine larvae is of limited value as evidence for virulence.

Challenge studies can be divided according to the method of administration of pathogens, depending on whether oral challenge or bath challenge has been used. Bath challenge assays involve bathing the target organism in a suspension of bacterial or viral pathogen (Hansen & Olafsen 1989). Challenge experiments with unfed fish larvae (i.e. yolk sac larvae) are today often conducted in multiwell assays (Bergh *et al.* 1992, 1997; Hjelm *et al.* 2004; Sandlund & Bergh 2008; Sandlund *et al.* 2010). A multiwell assay is relatively simple, provides a high number of replicates with one larva per well. However, such assays are limited to administration by bath, and must be terminated at the point of first feeding. Oral challenge of fish larvae during the first feeding stages includes experimentally manipulating a model food chain. Muroga *et al.* (1990) used bioencapsulation in rotifers and brine shrimp, followed by uptake in flounder (*Paralichthys olivaecus*) larvae, with subsequent development of pathology. Such oral challenge methods have been further developed by various research groups (Gatesoupe 1994, 1997; Munro *et al.* 1995; Grisez *et al.* 1996; Planas *et al.* 2006). In addition to virulence studies, such methods have also been used for efficacy studies with probiotics (Makridis *et al.* 2000a, 2001; Planas *et al.* 2006) and immunostimulants (Skjermo & Bergh 2004).

Challenge experiments need to be complemented by other methods in order to provide information on processes related to virulence, such as adhesion, penetration and proliferation in larval tissues. Histological methods, supplemented with tools that can identify the pathogen, such as immunofluorescence and immunohistochemistry can provide such information. Uptake and processing of *Vibrio anguillarum* in fish larvae have been investigated by immunohistochemistry in turbot (*Scophthalmus maximus*) (Grisez *et al.* 1996), halibut (Sandlund *et al.* 2010) and in cod larvae (Engelsen *et al.* 2008).

In situ methods are means to verify the presence of the pathogen in given larval tissues. *In situ* hybridization techniques detect not only the presence of a pathogen, but the active transcription of its genes. In challenges with viral pathogens, this method provides important additional information, as evidence of viral replication. For instance, Biering and Bergh (1997) studied infectious pancreatic necrosis virus (IPNV) in halibut, using immunohistochemistry to detect the presence of the virus in different larval organs, and *in situ* hybridization to detect transcription, hence viral replication. Sequential sampling, followed by histological and immunohistochemical processing of the sections can provide similar information on uptake, spreading and proliferation within the host. Grotmol *et al.* (1999) used immunohistochemistry of sequentially sampled individuals to describe the uptake and pathogenesis of nodavirus infections in halibut yolk sac larvae, demonstrating intestinal uptake, followed by spreading to different parts of the central nervous system.

Use of transformed bacteria with fluorescent reporter genes combined with methods such as confocal microscopy of whole larvae has been suggested by several researchers. Such methods could provide higher sensitivity than immunofluorescence, and could also be adapted to viral pathogens. Finally, the above mentioned methodological approaches can in the future eventually be combined with a gnotobiotic challenge, such as recently described for *Vibrio anguillarum* in seabass (Dierckens *et al.* 2009), to provide more rigorously controlled experimental conditions.

Immunology of fish larvae

Evolutionary aspects of innate immunity in fish

Teleostei is one of three infraclasses in the class Actinopterygii, which roughly makes up half of the extant vertebrate species, with high biodiversity, and they occupy a key evolutionary position in the development of immune responses. They are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity. It is known that early fish protection is achieved through a mixed passive immunity transmitted from maternal sources to offspring during vitellogenesis and oogenesis (Mulero *et al.* 2007a; Swain & Nayak 2009). In most fish, passive immunity is characterized by the presence of lytic enzymes, mainly lysozyme, and adaptive factors such as hormones and immunoglobulin. However, many of these maternally transferred factors usually persist only for a very limited duration and completely disappear thereafter in the late larval stage (Hanif *et al.* 2004), when the adaptive system can correctly operate. Thus, it is well recognized that along all the early stages of fish development, innate immunity plays a continuous role in orchestrating quick immune responses and protects larvae against the hostile environment, even when

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their own immunological capacity is still severely limited. In regard to fish larvae, innate defences are of particular commercial relevance. In many important cultured species such as the Mediterranean gilthead sea bream (*Sparus aurata*) and European sea bass, a delayed maturation of lymphomyeloid organs is observed (Scapigliati *et al.* 1995; Hanif *et al.* 2004). Delayed production of thymocytes makes fish unable to synthesize specific antibodies until several weeks after hatching (Swain & Nayak 2009). To overcome this immunological limitation, the evolutionary processes have provided fish innate immunity with wide capacities and mechanisms of protection that are activated just after egg fecundation, and become fully functional by the time of hatching. Thus, protection in developing embryos and further larval stages is driven by a complex network of innate defence mechanisms that include but are not limited to physical barriers, humoral factors, cellular defences and inflammatory processes (Secombes & Fletcher 1992; Zapata *et al.* 1996; Mulero & Meseguer 1998; Bols *et al.* 2001; Ellis 2001; Galindo-Villegas & Hosokawa 2005; Whyte 2007; Aoki *et al.* 2008).

Physical barriers, the first line of defence

The mucosal tissues, including mucus, epithelial cells lining the skin, gills and digestive tract of fish larvae, provide an initial barrier to the entry of disease-causing microorganisms like bacteria and viruses (Press & Evensen 1999). It is generally accepted that physical barriers are critical in maintaining osmotic equilibrium, in avoiding invading pathogens reaching the circulation and in triggering an extensive network of secondary innate immune responses. Several studies have been conducted on the nature and functionality of mucosal secretion. Dezfuli *et al.* (2002) reported that a mucous blanket is secreted from goblet cells in the epithelium immediately after the first contact with pathogens. However, it was only recently demonstrated through histological, histochemical and biochemical techniques that carp skin mucosa responds rapidly to high bacterial loads, even if the bacteria involved are considered to be non-pathogenic, with an increased secretion of mucin molecules, especially low glycosylated ones (van der Marel *et al.* 2010). Leknes (2010) recently reported that mucin-type molecules excreted by intestinal goblet cells seem to be highly species-specific, e.g. sulphated mucins in shi drum (*Umbrina cirrosa*), or neutral mucins in common dentex (*Dentex dentex*), both belonging to the order of Perciforms.

Among the physical barriers is the digestive tract, which, in addition to being a barrier against infections and environmental toxins, is actively involved in digestion, nutrient absorption, hormone secretion and immune function. Therefore, plasticity of this barrier shall be high and cells making up the gut must be highly specialized to discriminate among the huge amount of antigens that a fish may encounter throughout its life. Interestingly, Rawls *et al.* (2004) found that the molecular foundations of host–microbe interactions in zebrafish have a profound impact on larvae raised under germ-free conditions, manifested as decreased epithelial renewal and altered enterocyte morphology. Furthermore, in the teleost embryo, the close interaction of the gills with the external environment renders them susceptible to infection and they can serve as portals of entry for most bacterial pathogens. It is worthwhile remembering that thymus also develops from the anterior part of the digestive tract or pharynx, and the developmental processes of the digestive tract and the gills are indirectly linked, because the gill arches develop at the anterior part of the digestive tract, the pharynx. The innate immune system of epidermal structures relies on a wide diversity of immune cells and several biologically active glycoproteins strategically released in tissues directly exposed to the environment. Antibacterial peptides, complement proteins, lectins and pentraxins are among the most powerful biological components included in the previously mentioned structures (Gomez & Balcázar 2008).

Professional phagocytes and other myeloid cells

Fish immune cells show the same main features as those of other vertebrates, and lymphoid and myeloid cell families have been determined. Innate cellular defences of fish involve a variety of leukocytes, such as mononuclear phagocytes, polymorphonuclear leukocytes, and nonspecific cytotoxic cells (Buoncore & Scapigliati 2009). The so-called professional phagocytes are among the most intensively studied immune cells in fish and comprise the granulocytes, functionally equivalent to the neutrophils of higher vertebrates, macrophages, and lymphocytes. The cellular component exhibits interesting features such as the capacity to quickly start recognition of danger signals (either or not associated to molecular patterns), migration to the site in which relevant threats are detected and response using different strategies to fix any disruption of immunological significance (Galindo-Villegas *et al.* 2006). Studies on mobile phagocytes found in blood and secondary lymphoid tissue established the particular importance of these cells in inflammation, which is the characteristic cell response to microbial invasion and/or tissue injury leading to the local accumulation of leukocytes and fluid (Secombes 1996). Recently, innate cellular mechanisms have been elegantly demonstrated in real time using transgenic zebrafish larvae with green or red fluorescent protein-expressing myeloid cell lines, allowing

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the *in vivo* assessment of myeloid cell development and function, including recruitment to sites of tissue injury (Renshaw *et al.* 2006; Hall *et al.* 2007, 2009; Martin & Renshaw 2009). Regarding the ontogeny and morphology of immune cells and lymphomyeloid organs (thymus, spleen, head kidney and the gut associated lymphoid tissue), early studies have been conducted in several teleost species using mainly light and/or electron microscopy (Ellis 1977; Grace & Manning 1980; Botham & Manning 1981; O'Neill 1989; Chantanachookhin *et al.* 1991; Meseguer *et al.* 1991; Nakanishi 1991; Jósefsson & Tatner 1993; Quesada *et al.* 1994; Padrós & Crespo 1996; Zapata *et al.* 1997; Liu *et al.* 2004; Patel *et al.* 2009). Although informative, these methods rely upon researchers' abilities to look for key differences among related cell types. As a consequence, the development of comparative immunology in fish has been hampered by the lack of appropriate markers unequivocally to identify, isolate and functionally characterize the different immune cell types present in different species. In this way, several difficulties in producing effective antibodies against fish professional phagocytes have been identified, and for a long time only antibodies against macrophages from common carp (Rombout *et al.* 1993) and rainbow trout (Köllner *et al.* 2001) were available. However, most of these antibodies have been shown to recognize other immune cells different from the main target as well. So far, two highly specific antibodies have been generated that unequivocally recognize both professional phagocytes, acidophilic granulocytes (Sepulcre *et al.* 2002) and macrophages (Mulero *et al.* 2008b) of the gilthead sea bream. Using these innate cellular probes (G7 and Mcsf, respectively) in microscopical, immunohistochemical and automated flow cytometric analyses allowed correctly to establish the distribution, localization and relative abundance of positive cells during the ontogeny of lymphomyeloid organs in gilthead sea bream. Together with well-established techniques, these novel tools provided a unique approach to demonstrate that although the basic developmental mechanisms of teleosts are largely similar, there are differences with respect to the timing of developmental events and with respect to the role each professional phagocyte is playing.

Signaling in pattern recognition

The responses of innate immunity cells are driven by a diverse array of pattern recognition receptors (PRRs) that bind molecular motifs, conserved within all classes of microbes. However, these structures are widely known as 'pathogen-associated molecular patterns' (PAMPs) as they have traditionally only been associated with pathogens. PAMPs are recognized by C-type lectins, complement receptors, scavenger receptors and Toll-like receptors (TLRs), in all classes of fish studied so far. Very recently, it has been demonstrated that, in mammals, cellular injury can release endogenous 'damage-associated molecular patterns' (DAMPs) that also activate innate immunity (Zhang *et al.* 2010). The activation of a similar response by DAMPs and PAMPs could be explained through evolutionary processes in which mitochondria were evolved from endosymbionts that in turn were derived from bacteria (Sagan 1967) and so might bear bacterial, non-pathogenic, molecular motifs (e.g. the CpG repeats contained in mitochondrial DNA). Therefore, it would be expected that any cellular disruption by trauma, releases mitochondrial DAMPs into the circulation with potential to achieve a high concentration and trigger an uncontrolled downstream innate immune signaling response. It might be interesting to test whether the recognition of endogenous danger signals is conserved in fish and how the recognition mechanisms affect fish innate immunity.

PRRs specificity in fish

Fish innate immunity signaling pathways show a higher level of complexity than those in mammals. Zebrafish genes corresponding to many mammalian PRRs, have been identified and are often present with several paralogs. For example, zebrafish show about 50% more diversity in TLRs than mammals, most likely a consequence of the early genome duplication of the teleost lineage. As a consequence, this may allow for the recognition of a larger repertoire of PAMPs, or for more specific responses to individual molecular motifs. Furthermore, there is evidence that not all zebrafish TLRs have the same PAMP specificity or signaling activity as their mammalian counterparts. To point-out this feature, it has been reported recently for the first time the positive identification of TLR4 as a negative regulator of TLR signaling in fish, explaining in part the strange resistance of fish to the toxic effects of bacterial lipopolysaccharide (LPS) and the high concentrations of this substance required to activate the immune cells of these animals (Sepulcre *et al.* 2009).

Toll-like receptors

Toll-like receptors (TLR) are a highly conserved multigenic family which comprises several transmembrane and endosomal proteins first described in *Drosophila* (Anderson *et al.* 1985). In fish (as in mammals) these receptors are mainly involved in host defence, and TLR family members share the same structure, defined by the presence of leucine-rich repeats in their extracellular domain and by the presence of a Toll/IL-1 receptor domain in the *C*-terminal, the cytosolic part of the protein that initiates signal transduction (Sepulcre *et al.* 2009). TLRs recognize a variety of highly conserved pathogen-associated

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molecular patterns and play a pivotal role in modulating the immune response against invading pathogens. The interaction of a given PAMP with its specific TLR activates a signaling cascade leading to the expression of inflammatory cytokines and chemokines and to the activation of antimicrobial host defence mechanisms, such as the production of reactive nitrogen and oxygen radicals and antimicrobial peptides (Fig. 2). To date, 17 teleost TLRs (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) have been identified by genome and transcriptome analysis and remarkably distinct features of the TLR cascades among fish species have been reported. Most of this information is summarized in four major reviews about piscine TLRs (Bricknell & Dalmo 2005; Rebl *et al.* 2010; Takano *et al.* 2010; Palti 2011).

TLRs in larval fish

Recent molecular evidence has clearly identified the presence of TLRs in more than a dozen teleost species (Palti 2011). However, information on the functional ontogeny is limited. To clarify this issue, some laboratory trials have been conducted at the embryo and larval stages on selected fish species. Results of such trials successfully established the TLRs timing of functionality in the following species: zebrafish TLR 3, 4, 5 and 8 in the first 1–3 days post fertilization (Maijer *et al.* 2004; Phelan *et al.* 2005; Sepulcre *et al.* 2009; Stockhammer *et al.* 2009); rainbow trout TLR 5S all along the early ontogeny (Li *et al.* 2011), and carp TLR2 throughout the whole embryonic developmental stages (Samanta *et al.* 2012). Together, these results demonstrate that most TLRs signalling mechanisms start their functional activity mostly in the first few hours after fecundation which indicates the important role these PRRs play in fish larva immune surveillance.

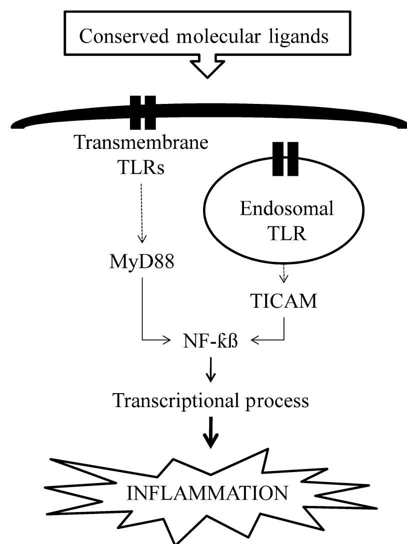

Figure 2 Simplified scheme of fish innate immune TLRs signaling pathways. Fish detects microbial invaders through highly specific pattern recognition receptors, called TLRs. Fish TLRs according to their transmembrane or endosomal location recruit the adaptor protein MyD88 or TICAM, respectively, to activate the master regulator NF-κß leading to the transcription of several potent inflammatory mediators. Toll-like receptors (TLRs), myeloid differentiation primary response gene (MyD88), Toll-like receptor adaptor molecule (TICAM).

Inflammatory cytokines and antimicrobial responses

Cytokines are the key regulators of the immune system. Their role in initiating inflammatory events in response to bacterial exposure is well-known in mammals, where a cytokine cascade leads to attraction of particular leukocytes and activation of their antimicrobial pathways. Tumor necrosis factor-alpha (TNF-α) is the first cytokine released in this cascade and leads to the downstream expression of interleukin-1β (IL-1β) and several chemokines (Secombes *et al.* 2009). Following infection with viruses, cytokines can activate again various cellular pathways but also have direct effects on cells that lead to an antiviral state, as seen with the interferons (López-Muňoz *et al.* 2009). In fish, such molecules represent a crucial component of the innate defences, although cross-talk and activation of adaptive immunity may also be triggered in the medium term. However, detailed studies should be conducted, mostly in molecular biology but including functional *in vivo* analyses, before making definitive conclusions. In the past few years, a huge increase in the knowledge of the cytokine network has been reported, mainly based on cDNA libraries, EST, and sequenced fish genomes of pufferfish, green spotted pufferfish, medaka, stickleback and zebrafish. Thus, applying homology cloning as the main tool, specific cytokines in different species has been described (Zou *et al.* 1999; Pelegrín *et al.* 2001; Garcia-Castillo *et al.* 2002; Corripio-Miyar *et al.* 2007; Roca *et al.* 2008).

Insiders of immunity in Teleost fish, the mast cells

Recent evidence from several laboratories has demonstrated that mast cells (MCs) are a key cell type of the haematopoietic lineage that has evolutionary conserved functions in pathogen surveillance (Abraham & St John 2010). The presence of MCs has been reported in all classes of vertebrates, including fish, amphibians, reptiles, birds, and mammals. Furthermore, current observations in fish point out the crucial location of MCs in connective tissue such as the gill filaments and the intestinal submucosa layer, suggesting these cells have an active task on pathogen recognition or other signs of infection (Reite

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& Evensen 2006; Mulero *et al.* 2007b; Dezfuli *et al.* 2010). Just recently, fish MCs have been noted to respond by migration and degranulation (Fig. 3) (Mulero *et al.* 2008a), giving support to previous suppositions that MCs were involved in host defence mechanisms in teleosts and are critical in fighting many infectious diseases (Reite 1997). In zebrafish larvae, beginning at 24 h post-fertilization, a significant role of MCs through the expression of carboxypeptidase A5 (cpa5), a MC-specific enzyme, has been observed in haematopoietic cells located at the anterior lateral paraxial mesoderm and in smaller numbers around the intermediate cell mass (Dobsson *et al.* 2008). However, a definite functional characterization of fish MCs has not been published yet. So far, fish MCs constitute a heterogeneous cell population exemplified by their heterogeneous morphology, granular content, sensitivity to fixatives and response to drugs (Crivellato & Ribatti 2010). Nevertheless, a controversial aspect in different fish species is the staining properties of the cytoplasmic granules of MCs, which are frequently described as either basophilic or eosinophilic, a characteristic expressed also by some amphibian and reptile MCs (Sottovia-Filho & Taga 1973). This characteristic has made different authors refer to them as mast cells, basophilic granular cells or acidophilic/eosinophilic granular cells. Besides basic similarities, granules of fish MCs were believed until few years ago to contain components common to their mammalian counterparts such as antimicrobial peptides, lytic enzymes or serotonin, but lack the presence of histamine. Mulero *et al.* (2008b) attempted to clarify this controversy using the monoclonal antibody G7 specific to acidophilic granulocytes (AGs) (Sepulcre *et al.* 2002) in gilthead sea bream. From the negative fraction of the analysed population, two different eosinophilic cell types were found in connective tissue, although neither eosinophilic cell types nor acidophilic granulocytes of gilthead seabream showed the metachromatic staining characteristic of mammalian MCs after being stained with toluidine blue at low pH. Using antibodies and immunostaining techniques, Mulero *et al.* (2007b) demonstrated the presence of histamine in fish MCs. Surprisingly, this was true only in species belonging to the largest and most evolutionary advanced order of teleosts, the Perciformes. Furthermore, functional studies indicated that fish professional phagocyte function may be regulated by the release of histamine from MCs upon H1 and H2 receptor engagement. These observations indicate that histamine is biologically active and can regulate the inflammatory response of fish by acting on professional phagocyte signaling. Recently, in mammalian MCs, many signaling mediators upstream of degranulation have been identified and these converge to a common requirement for generating a Ca^{2+} flux into the responding cell (Kalesnikoff & Galli 2008). A similar pathway has not been reported in fish so far.

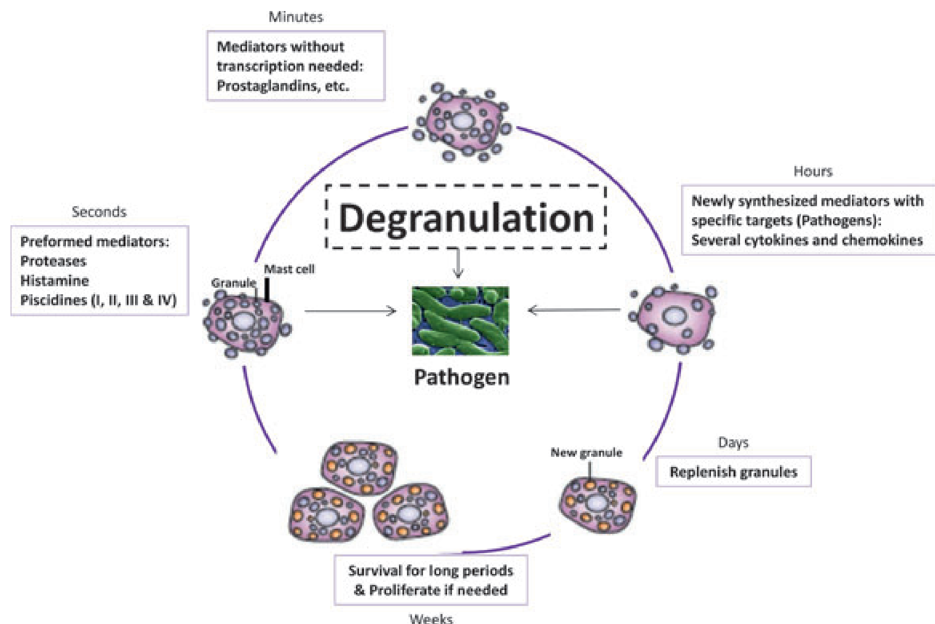

Figure 3 Degranulation of mast cells in response to pathogens. Upon activation by pathogens, mast cells migrate to specific tissues to undergo degranulation of preformed or newly synthesized products showing specific time patterns and increasing target specificity, which ultimately lead to mast cell survival and proliferation.

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Mast cell antimicrobial peptides: the piscidins

Antimicrobial peptides (AMPs) are host defence effector molecules that probably occur in all life forms, and starting from birth play a key role in fish innate immunity. Since long ago, they have been reported effectively to combat infections caused by viruses, bacteria, fungi and parasites (Yano 1996). However, recently, an increasing number of AMPs produced by fish (e.g. paradoxins, pleurocidins or hepcidins) have been reported to be able to act as general disinfectants. Antimicrobial peptides are typically present in fish mucosal surfaces and skin, which represent major routes of entry of pathogens (see Smith and Fernandes (2009) for a review). Silphaduang and Noga (2001) reported for the first time the successful isolation of a cationic piscidins from Acanthopterygii fish MCs through modified acid-urea polyacrylamide-gel electrophoresis. Initially, the family of piscidins comprised three broad-spectrum AMPs (piscidin 1, 2 and 3), which are 22 amino acids long and have a highly conserved, histidine- and phenylalanine-rich *N*-terminus combined with a much more variable *C*-terminus. Just recently, a new member of the piscidins family, piscidin 4, was isolated and characterized (Noga *et al.* 2009). All type of piscidins known so far, disinfect exposed surfaces and kill microorganisms that could enter the host through injuries, preventing them from proliferating until they are sequestered and eliminated by the phagocytes and other components of the systemic immune system. So far, piscidines have been immunolocalized in healthy and infected MCs of the skin, gills, gut and lining blood vessels in the viscera (Dezfuli & Giari 2008; Dezfuli *et al.* 2010). Thus, it can be expected that among AMPs, piscidins shall be a fundamental innate strategy in controlling host–microbial interactions at the early larval stages.

The ontogeny of the adaptive immune system

The study of the ontogeny of the immune system in fish is relevant for two reasons. First, the comparison of the ontogeny of the fish immune system with that of higher Vertebrates offers new insights into developmental biology in general, and more specifically, the generation of a functional immune system. Teleost fish are a unique biological model for studies on the development of immune system of Vertebrate species, since they have larval stages during which they rely solely on innate responses, a transition phase in which components of acquired responses appear, and an 'adult' stage characterized by fully functional innate and acquired responses. Secondly, knowledge of the developmental sequence of immune function is imperative to design preventative measures against problems concerning infectious diseases in aquaculture, in particular to avoid vaccination at a too early stage. A variety of haematopoietic genes have been sequenced, generating a more detailed picture of the development of the distinct leukocytes and their precursors. Despite this information, basic questions including the identification of the first lympho-haematopoietic sites, the origin of T- and B-lymphocytes and the acquisition of full immunological capacities remain to be resolved (Zapata *et al.* 2006).

Maternal transfer of IgM

It is known that mature eggs of fish species contain IgM (Olsen & Press 1997; Seppola *et al.* 2009), although the presence of antibody molecules related to a transfer of specific immunity is still a matter of speculation (Hanif *et al.* 2004; Haines *et al.* 2005). Previous studies revealed that in sea bream, fluctuations of serum IgM concentration relate to the onset of the reproductive cycle and to gender, and could be detected in the released eggs (Picchietti *et al.* 2001). In mature sea bass eggs, Ig protein was detected corresponding to 6 $\mu g\ g^{-1}$ egg weight and immunocytochemical analysis of paraffin sections from 5-day post-hatched embryos revealed an accumulation of material immunoreactive with the anti-IgM monoclonal antibody DLIg3 in the yolk sac (Scapigliati *et al.* 1999), as well as IgM gene transcripts in ovarian follicles throughout oogenesis (Picchietti *et al.* 2004). After fertilization, the mRNA coding for IgM disappears from sea bass embryos after 3–4 days, and IgM transcripts start to be detected again after 45 days. From these studies, it is evident that sea bass and sea bream transfer IgM to the egg through active processes, and that IgM undergo a rapid decay after fertilization. There is no clear understanding on the IgM presence in fish eggs, and considering their amount reported above, it is reasonable to speculate that unspecific IgM could be useful as antigen-opsonizing molecules during early stages of sea bass development. To reinforce this latter hypothesis, it should be remembered that fish eggs also contain complement system components (Huttenhuis *et al.* 2006c; Løvoll *et al.* 2006).

Development of T cells and T cell-associated molecules

The knowledge on the ontogenetic development of lymphocytes has been acquired in carp (Huttenhuis *et al.* 2006a,b), but mainly in sea bass for marine species for which monoclonal antibodies are available for thymocytes and peripheral T cells (Scapigliati *et al.* 1995) and for IgM and B cells (Scapigliati *et al.* 1996). The combined use of these cellular probes permitted to define the timing of appearance of lymphocytes during ontogenic development of sea bass, extensively reviewed in a previous work (Rombout *et al.* 2005). Indeed, by using these antibodies in immunohistochemistry of sea bass larval sections, a

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dramatic difference in the timing of the appearance of lymphocytes emerged, with T cells preceding B cells by about 20 days (at 18°C).

Recently, Picchietti *et al.* (2008, 2009) applied RT-PCR to investigate in sea bass the developmental appearance of TCRβ, CD8α and CD4, three important genes involved in T cell functions (keeping in mind that in general, gene expression does not always mean that a complex system is functional). For this purpose, total RNA was extracted from released eggs and pools of specimens sampled at different time points (2, 4, 8, 13, 16, 21, 25, 32, 51, 62, 75 and 92 days post-hatch) and from the thymus of 1 year-old specimens, reared at 15 ± 1°C. RT-PCR detected the first appearance of TCRβ in larvae between 21 and 25 days post-hatch, which is well before the first expression of CD8α and CD4 (i.e. between 40 and 50 days post hatch). The early *TcRβ* expression confirmed data obtained in other Teleost species such as rainbow trout (*Oncorhynchus mykiss*) (Hansen & Zapata 1998; Fischer *et al.* 2005) and was in concert with the early development of the thymus, which is the first organ to become lymphoid in Teleosts (Botham & Manning 1981; Abelli *et al.* 1996; Padrós & Crespo 1996; Schrøder *et al.* 1998). Further, the expression of *CD8α* and *CD4* was detected in sea bass between 40 and 50 days post-hatch (Picchietti *et al.* 2008, 2009), when the thymus is characterized by a distinctive lymphoid composition (Abelli *et al.* 1996) whose normal development is dependent upon cell interactions with thymic stroma and secreted molecules (Zapata *et al.* 1997) as previously reported in Mammals (Maddox *et al.* 1987). In sea bass, real time PCR also demonstrated that transcripts of TCRβ, CD8α and CD4 increased until 92 dph. At this stage, the TCRβ and CD8α transcripts were significantly increased compared with all previous stages, while CD4 transcripts were significantly higher when compared with day 25 and day 51 post-hatch, suggesting that the critical events of differentiation and selection of T-lymphocytes are strongly correlated to the histological maturation of the sea bass thymus. In addition, in the thymus of 1-year-old sea bass, the amounts of CD4 and CD8 transcripts are not statistically different, while CD4 transcripts are significantly lower than TCRβ transcripts, suggesting that a higher number of TCRβ^+ thymocytes than CD4$^+$ subpopulations could be located in the thymus, or that a fraction of CD8$^+$ thymocytes could express different TCR chains (most likely $\gamma\delta$).

In situ hybridization with digoxigenin (DIG)-labeled RNA probes showed that at 51 days post-hatch the TCRβ, CD8α and CD4 mRNAs were localized in thymocytes of the outer and lateral zones of the thymic paired glands that were not yet lobulated, while the parenchyma was regionalized. Already at day 51 and from day 75 post-hatch on, an active lymphopoiesis was observed in the gland resulting in a cortex/medulla demarcation in which the signal was restricted to cortical thymocytes (Fig. 4). At 92 days post-hatch, the histology of the thymus was well established and TCRβ^+, CD8α^+ and CD4$^+$ cells were mainly localized in the cortex at the cortico-medullary junction, providing new data on the understanding of the thymic microenvironment and evidence relating to the positive selection and differentiation of T cells.

Recently Øvergård *et al.* (2011) investigated the expression of RAG1, TCRα, TCRβ, CD3$\gamma\delta$, CD3ϵ, CD3ζ, CD4, CD4-2, CD8α, CD8β, Lck and ZAP-70 in larval and juvenile stages of Atlantic halibut. An increase in mRNA transcripts for the genes was seen at different time points, from 38 days post-hatching (dph) about the time when the first anlage of thymus is found, and onwards. The transcription patterns of the 12 mRNAs were found to be similar throughout the developmental stages tested. *In situ* hybridization on larval cross-sections showed that

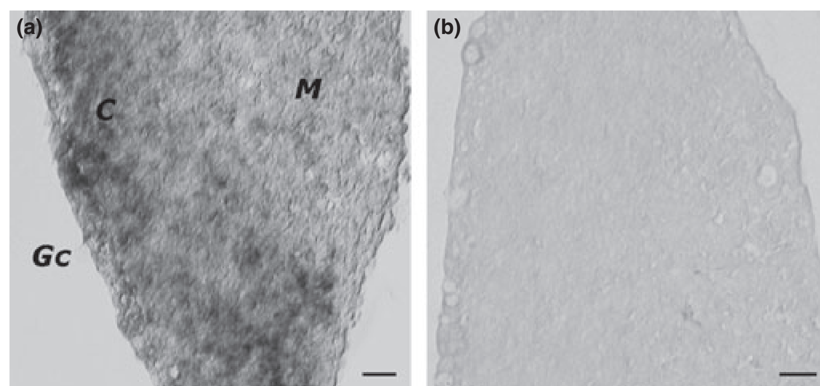

Figure 4 TCRβ *in situ* hybridization of developing thymus aged 75 days post-hatch. (a) TCRβ^+ cells were clearly concentrated in cortical region. Bar = 20 μm. (b) Negative control of ISH with a sense RNA probe. Bar = 20 μm. GC, gill chamber; C, cortex; M, medulla.

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RAG1 and Lck could be detected in lymphocyte-like cells within the thymus at 42 dph. CD4 expression could not be detected within the thymus before 66 dph, however, positive cells were restricted to the cortical region. At 87 dph, the zonation of the thymus in a cortical, cortico-medullary, and a medullary region seemed to be more evident with CD8α expressing cells found in all regions, indicating the presence of mature T-cells. This correlates with previous results by Patel *et al.* (2009), describing thymus development and the appearance of IGM+ cells during halibut ontogenesis.

In teleosts, nothing is known yet about the origin of lymphoid progenitors that colonize the thymic rudiment, while a new hypothesis of positive selection and differentiation of T cells was recently proposed in sea bass juveniles (Picchietti *et al.* 2008, 2009). At this stage, the thymus retained a superficial localization and was more extensively lobulated when compared with previous developmental stages. TCRβ, CD8α and CD4 expression was detected by *in situ* hybridization in the cortex and at the cortico-medullary junction, in each lobe of juveniles, evidencing the compartmentalization of the parenchyma in which a network of stromal epithelial cells was established and in the meshes of which thymocytes are located. The regions housing CD8+ and CD4+ cells were largely overlapping. In fact, their density was nearly equal, suggesting that the cortex could be the site of double positive cells. CD4 and CD8 thymocytes were significantly less numerous in the medulla than in the cortex, while their relative numbers were not different in the medulla. In this district, large cords of $CD8^+$ cells extended in the gland, whereas CD4 transcripts were detected in isolated thymocytes reflecting the occurrence of SP single positive thymocytes in the medulla. Furthermore, the presence of a $CD8\alpha^-$, $CD4^-$ and $TCR\beta^+$ subcapsular lymphoid zone is worth noting, where the thymocytes do not express the two co-receptors and thus can be regarded as double negative (DN). This pattern of localization of thymic subsets in distinct anatomical compartments is consistent with an out and in pattern of thymocyte migration. As evidenced in mammals, each of the T-cell maturation events takes place in a discrete region of the thymus and relies on interactions with specialized resident cells found in each of these anatomical regions (Ladi *et al.* 2006). Although thymocyte migrations are not defined in teleosts, the data suggested high similarity in the morpho-functional organization of the teleost thymus compared with mammals. An interesting finding was the lack of a $CD8\alpha^-$ lymphoid zone in the cortical thymic parenchyma contacting the pharyngeal epithelium which is related to the absence of a connective capsule (Abelli *et al.* 1996) and hence to the lack of limiting epithelial cells that likely play a role in attracting double negative thymocytes.

Taken together, from data available in the literature it can be evidenced that each fish species possesses its own ontogenetic developmental pattern, likely related to evolutionary-acquired habits (Langenau & Zon 2005; Huttenhuis *et al.* 2006b,c; Zapata *et al.* 2006; Corripio-Miyar *et al.* 2007; Patel *et al.* 2009) and, importantly, it has been confirmed in fish that T cell development precedes B cell development. Based on the available data it is conceivable to assume that in sea bass the acquired immune system, in its main known components, namely lymphocytes bearing TcRs or IgM, MHC, RAGs and T cell coreceptors could be ready to perform full activities from day 45 post-hatching onwards.

Steering larval microbial communities to the benefit of the host

The information in relation to microbial community composition (including pathogens) and activity on one hand and the developing immune system on the other hand has progressed considerably the last two decades, but a comprehensive insight is still lacking. In particular, there is ample room for scientific advancements if research fields such as larval microbiology and larval immunology could interact. Despite the lack of sufficient and sound tools (both at the microbiological and immunological level), a vast amount of literature is available on microbial interference with the host, e.g. at the level of microbial contribution to larval nutrition and on the effect of probiotics and prebiotics.

Microbial contributions to larval nutrition and physiology

The beneficial contribution of bacteria to digestion in fish was first acknowledged with the fermentative processes that occur in the long intestine of some species, especially in warm waters (Clements 1997). The nutritional importance of microbiota in the short and immature intestine of fish larvae seemed less obvious. The amount of suspended bacteria ingested by carp larvae was estimated to be 10^5–10^6 cells per individual within 45 min (Matena *et al.* 1995), which may support the assumption of a direct nutritive contribution of bacterioplankton. However, the data of Reitan *et al.* (1998) suggest ingestion rates of bacteria by turbot larvae to be 1–3 orders of magnitude lower. These and other early speculations were reviewed by Hansen and Olafsen (1999) and Ringø and Birkbeck (1999). However, it was not proven that fish larvae could digest bacteria in the intestinal lumen. Olafsen and Hansen (1992) observed the uptake of bacteria in enterocytes of cod and herring larvae, and they hypothesized that such endocytosis could provide some

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exogenous nutrients at the yolk sac stage, besides its role in immune stimulation. Another hypothesis was the contribution of bacterial enzymes to the digestive process, but this is difficult to evaluate in most cases. The fact that bacteria are rich in mineral nutrients has so far not been considered (Vadstein 2000).

The essential role of the associated microbiota on early gut development and nutrient metabolism in fish larvae is evident in gnotobiotic zebrafish. Rawls *et al.* (2004) showed that intestinal epithelial proliferation and gene expression were modulated depending on the presence and composition of the gut microbiota. These authors compared fish reared conventionally, in axenic conditions, and after re-contamination of the bacteria-free larvae with 'conventional' microbiota. Briefly, enterocyte proliferation in germ-free larvae was lower than in those reared with microbiota. Some transcriptional responses of the axenic animals were similar to those caused by fasting, with respect to fatty acid oxidation and neoglucogenesis. Genes involved in cholesterol metabolism and in amino acid transport were also affected by the absence of gut microbiota. A follow-up study (Rawls *et al.* 2006) showed that the entire microbial community induced the modulation of such gene expression, whereas the response was less marked with individual strains, bacterial consortia and murine intestinal microbiota. The influence of gut microbiota on gut differentiation in zebrafish was also studied by Bates *et al.* (2006), who found slow intestinal motility in germ-free animals and the maintenance of immature epithelial features in their gut, such as poor alkaline phosphatase activity and up-regulation of glycoconjugate expression. This impaired development could be amended by exposure to single strains of bacteria. The experiments of Rawls *et al.* (2004, 2006) provide the first insight in microbial interference in host gene expression and development. Experiments with gnotobiotic cod larvae confirm several of the observations from zebrafish, including microbial specificity of the host response and types of genes upregulated (Forberg *et al.* 2011b, 2012). These cod experiments have also demonstrated that also dead bacteria result in a significant upregulation of genes, but the difference between live and dead bacteria varies for genes dependent on functionality (Forberg *et al.* 2012). The types of responses found in zebrafish are partly confirmed in cod and are similar to those in mice, suggesting a broad evolutionary conserved response. However, it would be useful to perform more gene experiments aimed at boosting our knowledge on host–microbe interactions in commercially important aquaculture species, including both vertebrates and invertebrates. The ultimate goal would be to use a combination of zootechnical, nutritional, genetic and immunological tools. With respect to the experiments published by Rawls *et al.* (2004, 2006) further experimental improvement could be generated by the use of purified diets (Carvalho *et al.* 2006), rather than commercial diets. The latter might contain uncharacterized microbial cellular compounds that might have an effect on, for example, the innate immune response, blurring the early microbial response in the host.

In standard rearing conditions with the presence of 'conventional' microbiota, many beneficial effects caused by the microbiota have been documented, with impact on both fish health and nutrition. Practical consequences for survival, growth or development have been documented. For example, with the Microbial Maturation Concept (see 'Steering the microbial community composition and/or activity') several positive effects on larvae have been documented, including increased feeding rate, stress tolerance, survival and growth (Vadstein *et al.* 1993; Skjermo *et al.* 1997; Salvesen *et al.* 1999). Plante *et al.* (2007) noted that the administration of *Arthrobacter* sp. RSXII increased the level of reserves of lipids and essential fatty acids, and thus improved the nutritional status of haddock larvae. Some microbes seem particularly helpful to stimulate the digestive system in fish larvae. Tovar-Ramirez *et al.* (2002) showed that the onset of digestive enzyme activities in the enterocytic brush border membranes was accelerated in sea bass larvae fed a compound diet with live *Debaryomyces hansenii* HF1, but not in another group fed *Saccharomyces cerevisiae* X2180. This difference between the efficiency of both microorganisms was paralleled to their polyamine production. It has also been shown that purified spermine induced sea bass gut maturation (Péres *et al.* 1997). A compound diet containing 10^6 CFU g^{-1} of live *D. hansenii* improved growth, survival and conformation of sea bass larvae (Tovar-Ramirez *et al.* 2004). This dietary yeast also stimulated the protection system against oxidative stress in the larvae (Tovar-Ramírez *et al.* 2010). In rainbow trout fry *D. hansenii* naturally colonized the intestine during the first month of feeding, and an early artificial introduction of *S. cerevisiae* var. *boulardii* accelerated gut maturation (Waché *et al.* 2006). A commercial preparation of lactic acid bacteria also strengthened the activity of digestive enzymes in sea bream larvae (Suzer *et al.* 2008). Avella *et al.* (2010) investigated the metabolic pathways that could be modulated by the introduction of *Lactobacillus rhamnosus* during the larval rearing of clownfish. In particular, these authors analysed the regulation of the expression of some receptors involved in the pathways between nutrients and cell differentiation, which were reviewed elsewhere (Zambonino Infante & Cahu 2007). However, such molecular biomarkers should be interpreted cautiously. For example, the up-regulation of peroxisome proliferator-activated receptor α was regarded

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as a positive indicator of development in whole clownfish larvae by Avella *et al.* (2010), while Rawls *et al.* (2004) considered this as a sign of compromised ability to use nutrients in the digestive tract of zebrafish larvae.

Besides beneficial microbes and virulent pathogens, many strains may exhibit opportunistic behaviour, which can exert adverse effects due to, for example, competition for nutrients between the strains and the host. Iron supply is crucial in this interaction, and the presence of siderophores or siderophore-producing probiotics could reduce the incidence of opportunistic *Vibrio* sp. in sea bass larvae (Gatesoupe 1997). The composition of the diet should take into account the bacterial interference, in order to meet the requirement of the host, while avoiding iron overload (Gatesoupe 1997). The response of the host could be assessed by measuring the expression of genes encoding iron-binding proteins and hepdicin antimicrobial peptides, which seem related to the regulation of iron uptake in fish larvae (Andersen 1997; Martin-Antonio *et al.* 2009). Microbes can compete with the host for many other substrates, such as the peptides that are required by fish larvae, favouring the dominance of *Vibrio* sp. (Kotzamanis *et al.* 2007). Some microbes can also cause nutritional pathologies, such as *Paenibacillus thiaminolyticus*, whose thiaminase activity was suspected to cause early mortality in lake trout (*Salvelinus namaycush*), due to vitamin B_1 deficiency (Richter *et al.* 2009).

The interaction between microbiota and the nutrition/physiology of fish larvae appears complex, but the information available is still fragmentary. It is essential to further investigate the effects of dietary components and feeding methods with improvement of microbial management in fish hatchery as motivation. In that respect, the use of purified diets in combination with a gnotobiotic environment might be instrumental.

Steering the microbial community composition and/or activity

Steering microbial community composition

Several review articles have been written about the procedures to isolate probiotic micro-organisms (Gatesoupe 1999; Balcazar *et al.* 2006; Vine *et al.* 2006). They include isolation and verification *in vitro* of, for example, the possibility to inhibit the growth of a pathogen or the ability to attach or grow in mucus. However, these *in vitro* features neither ensure a positive effect *in vivo* (but should be verified) nor guarantee that the mode of action *in vivo* is related to the phenotypes described *in vitro* (Tinh *et al.* 2007a). In fact, little information is available on the mode of action of putative probiotics *in vivo*. In order to move away from the largely empirical approach in the identification and use of probiotic bacteria, novel tools should be employed. Those might include gnotobiotic models, gene expression in wild type and mutants, and gene expression in the host (e.g. those involved in innate immunity, cell proliferation or nutrient metabolism, see above).

Keeping in mind the shortcomings of much of the scientific research on probiotics in aquaculture, especially in relation to the interpretation of the mode of action *in vivo*, it is of interest to review the results reported thus far. The use of probiotic microorganisms in the rearing of marine fish larvae, molluscs and shrimps has been reported in several experimental studies in the past decade (Vine *et al.* 2006). The way of application in first life stages can be divided into three main approaches: addition of bacterial cultures to the water (Ringø & Vadstein 1998; Lauzon *et al.* 2010), encapsulation in the diet (Makridis *et al.* 2001; Planas *et al.* 2006; Swain & Nayak 2009) (or combined; Makridis *et al.* 2000a), and modulation of the microbiota in the biofilter (when such a device is applied in the experimental system) (Prol-Garcia *et al.* 2010). Addition of probiotics in the live food has a dual purpose: on one hand replacing opportunistic bacteria that normally proliferate in live food cultures and on the other hand, providing bacteria that have a beneficial effect on the larvae (Skjermo & Vadstein 1999). Bioencapsulation of bacteria in rotifers or brine shrimp offers the possibility to influence to a high degree the microbiota associated with live feed (Verschuere *et al.* 1999; Makridis *et al.* 2000b). The use of fresh cultures of probiotics and the possibility to bioencapsulate them in live feed have been shown in several studies directly to influence the microbiota of the reared larvae, and in several cases the larval microbiota was dominated by the added probiotic bacteria (Suzer *et al.* 2008). The dosing of probiotics at this stage is critical, as superfluous numbers of bacteria could accumulate in the rearing system and deteriorate the environmental conditions for the larvae. On the contrary, the addition of probiotic bacteria in the feed of postlarvae and juveniles, where the stomach barrier has already been established, has shown that the presence of bacteria in the gut is dependent on the continuous addition of bacteria via the feed, so that the true colonization of intestinal epithelium is under question (Makridis *et al.* 2008).

Another way of altering the composition of the intestinal microbial community is by using prebiotics, substrates that cannot be digested by the host but promote beneficial intestinal bacteria. The interest for prebiotics in larviculture is currently emerging (for reviews see Gatesoupe 2005; Ringø *et al.* 2010). Dietary oligofructose, for example, increased the growth rate of metamorphosing turbot, while changing the composition of the intestinal microbiota (Mahious *et al.* 2006). However, as research on the

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use of prebiotics for aquaculture is still in its infancy, more research is needed fully to conclude the effects of adding prebiotics in fish diets.

The third way of controlling the composition of the microbiota is by setting up selection regimes (Vadstein *et al.* 1993). The first attempts were by setting up a selection for K-strategists to avoid opportunists according to what has been named the Microbial Maturation Concept (Vadstein *et al.* 1993; Skjermo *et al.* 1997; Salvesen *et al.* 1999). This method secures a continuous supply of likely beneficial bacteria and thus circumvents some of the problems with how lasting the addition of probiotics is. Recently it has been proposed and tested that recirculated aquaculture systems also have positive effects on larvae through K-selection regimes on microbes mediated through the biofilters (Attramadal *et al.* 2012).

Steering microbial activity

Quorum sensing, bacterial cell-to-cell communication, is a candidate target to modulate microbial activity to the benefit of the host. The aquaculture pathogen *Vibrio harveyi* has been found to use a three-channel quorum sensing system, mediated by the signal molecules HAI-1, AI-2 and CAI-1, respectively (for a review see Defoirdt *et al.* 2008). The signals are detected at the cell surface by membrane-bound, two-component receptor proteins channeling the signal into a common phosphorylation/dephosphorylation signal transduction cascade (Taga & Bassler 2003). Phenotypes found to be controlled by this quorum sensing system *in vitro* include bioluminescence (Bassler *et al.* 1993) and the production of several virulence factors such as a type III secretion system (Henke & Bassler 2004), extracellular toxin (Manefield *et al.* 2000), metalloprotease (Mok *et al.* 2003), chitinase (Defoirdt *et al.* 2010) and a siderophore (Lilley & Bassler 2000). Making use of the possibility to culture brine shrimp (*Artemia franciscana*) larvae under gnotobiotic conditions, the importance of quorum sensing in relation to *Vibrio* virulence was verified. Here the gnotobiotic conditions provide the opportunity to eliminate interference eventually created by the production of quorum sensing molecules synthesized by microorganisms not under study. It was found that *Vibrio harveyi* inactivated in the AI-2 channel were considerably less virulent, whereas inactivation of the HAI-1 channel had no effect. Yet, in gnotobiotic rotifers (*Brachionus plicatilis*), in which the effect of *Vibrio* on the growth rate was measured, it appeared that the AI-1 and AI-2 system need to be inactivated simultaneously in order to mitigate the negative effect of the bacterium (Tinh *et al.* 2007b). These findings suggest that the way in which quorum sensing regulates the virulence of a specific pathogen might depend on the host. It also suggested that the involvement of quorum sensing in negative host–microbial interactions might be valid for other larval aquatic organisms of importance to aquaculture. Additional evidence was produced under non-gnotobiotic conditions in the larviculture of turbot and giant river prawn (*Macrobrachium rosenbergii*) (Tinh *et al.* 2008; Nhan *et al.* 2010). These authors could demonstrate that the addition of a mixture of *N*-acylhomoserine lactone quorum sensing molecules (daily addition of 1 mg L^{-1}) to the culture water had a detrimental effect on survival. Interestingly, this negative effect could be mitigated through the addition of a microbial community able to degrade the added quorum sensing molecules. So far, very few studies have been performed on the impact of quorum sensing on the virulence of fish pathogens. In one report, Rasch *et al.* (2004) showed that a quorum sensing-disrupting brominated furanone protected rainbow trout (*Oncorhynchus mykiss*) from vibriosis caused by *Vibrio anguillarum*. Moreover, quorum sensing regulation of virulence factor expression has been reported in many fish pathogens, including *Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, Vibrio anguillarum, V. ichthyoenteri, V. salmonicida* and *Yersinia ruckeri* (for a review, see Natrah *et al.* (2011)). In order to firmly establish the importance of quorum sensing in larval fish and shellfish disease, gnotobiotic model systems would be instrumental, e.g. allowing verifying the *in vivo* production of quorum sensing molecules at the moment of infection. For sea bass larvae, a gnotobiotic system has recently been described (Dierckens *et al.* 2009), also demonstrating that certain *Vibrio anguillarum* strains can cause accelerated mortality. Such zootechnical tools will definitely help to provide evidence for the involvement of quorum sensing in host–microbial interactions.

Acknowledgements

This work was supported in part by the cost action FAO801 LARVANET: Critical success factors for fish larval production in European Aquaculture: a multidisciplinary network, and by the European FP7 project ‘Promicrobe – Microbes as positive actors for more sustainable aquaculture’ (Project Reference: 227197), and by Research Foundation of Flanders (FWO) project ‘Probiont induced functional responses in aquatic organisms’.

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