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Immunotoxic effects of environmental toxicants in fish — how to assess them?

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Abstract Numerous environmental chemicals, both long-known toxicants such as persistent organic pollutants as well as emerging contaminants such as pharmaceuticals, are known to modulate immune parameters of wildlife species, what can have adverse consequences for the fitness of individuals including their capability to resist pathogen infections. Despite frequent field observations of impaired immunocompetence and increased disease incidence in contaminant-exposed wildlife populations, the potential relevance of immunotoxic effects for the ecological impact of chemicals is rarely considered in ecotoxicological risk assessment. A limiting factor in the assessment of immunotoxic effects might be the complexity of the immune system what makes it difficult (1) to select appropriate exposure and effect parameters out of the many immune parameters which could be measured, and (2) to evaluate the significance of the selected parameters for the overall fitness and immunocompetence of the organism. Here, we present — on the example of teleost fishes — a brief discussion of how to assess chemical impact on the immune system using parameters at different levels of complexity and integration: immune mediators, humoral immune effectors, cellular immune defenses, macroscopical and microscopical responses of lymphoid tissues and organs, and host resistance to pathogens. Importantly, adverse effects of chemicals on immunocompetence may be detectable only after immune system activation, e.g., after pathogen challenge, but not in the resting immune system of non-infected fish. Current limitations to further development and implementation of immunotoxicity assays and parameters in ecotoxicological risk assessment are not primarily due to technological constraints, but are related from insufficient knowledge of (1) possible modes of action in the immune system, (2) the importance of intra- and inter-species immune system variability for the response against chemical stressors, and (3) deficits in conceptual and mechanistic assessment of combination effects of chemicals and pathogens.

Keywords Immunotoxicity · immune system · ecotoxicological risk assessment · ecotoxicology · ecoimmunology · fish

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Introduction

Ecology investigates the processes that keep ecological systems functioning. Ecotoxicology deals with the risk of toxic chemicals to disrupt the functioning of ecological systems. Traditionally, ecotoxicology assesses the ecological impact of environmental toxicants by determining chemical effects on growth and reproduction of species, as changes of these parameters may translate into altered population density, although this translation is not a linear, deterministic process but is influenced by a number of extrinsic and intrinsic

processes (Newman 2001; Calow and Forbes 2003; Segner 2007, 2011a). Toxicant-induced alterations of population density are not only relevant for the directly impacted species but can indirectly affect other species. For instance, if a toxic chemical is lethal to a predator species, this will directly affect predator density, and indirectly prey species density (e.g., Fleeger et al. 2003). Importantly, species interactions are not only affected through chemical-induced changes in species density but also through effects on species traits (Relyea and Hoverman 2006). Particularly sublethal concentrations of toxicants which remain without overt effects on the density-related parameters, survival and reproduction, still may have consequences for species interactions by inducing changes of behavioural or physiological traits. An example of a trait-mediated effect would be chemical-induced altered foraging behaviour of a predator species which will then lead to altered density of the prey species (Weis et al. 2001; Relyea and Hoverman 2006). Another example of trait-mediated effects on species interactions are the effects of chemicals on host–pathogen systems: Exposure to toxic chemicals can impair the immunocompetence of the host species, so that the adverse ecological outcome results not from the chemical toxicity per se, but from the indirect effect of the chemical on the capacity of the host species to interact with and to resist to the pathogen. For assessing the consequences of chemical contamination for ecological systems, we have to consider both density- and trait-mediated toxic impacts (Relyea and Hoverman 2006).

A number of field studies have shown that the impact of chemical contamination on the immunocompetence on wildlife species bears ecological relevance (Luebke et al. 1997). Prominent examples come from studies on the causes of the global decline in amphibian populations, which showed that exposure to pollutants can lead to compromised immune function and increased infection rate with parasites (Kiesecker 2002; Rohr et al. 2008). Other examples include the outbreak of distemper virus in PCB-contaminated harbour seals and harbour porpoises (Beinecke et al. 2005; Mos et al. 2006*)* or the increased disease susceptibility and incidence in fishes from the polyaromatic hydrocarbon (PAH)-contaminated Puget Sound (Arkoosh et al. 1998, 2001). Also for invertebrate species, there exist a number of reports of increased disease incidence in populations from contaminated environments (Galloway and Depledge 2001).

Good immunocompetence is a critical fitness determinant, as in their natural environment organisms are constantly exposed to pathogens (Graham et al. 2010). The threat which pathogens impose on host species is evident from the fact that host–pathogen interactions are a major driver in the evolution of life histories. Good immunocompetence enables the individual to maintain good health so as to minimize the fitness costs of infection (Owen and Wilson 1999). Chemicals impair the immunocompetence of exposed organisms through a variety of mechanisms, including interference with signalling pathways in immune cells, suppressing immune functions such as oxidative burst activity, induction of apoptosis (Bols et al. 2001; Köllner et al. 2002; Reynaud and Deschaux 2006; Nakayama et al. 2009), but also through trade-offs between energetically costly immune defenses and the energy demands of the toxicant defense (cf. Segner et al. 2011). In ecology, the importance of the immune system in an organism's evolutionary, ecological and life history context is well recognized (Sheldon and Verhulst 1996; Demas et al. 2011), while in ecotoxicology there exists no corresponding awareness of the importance of a functional immune system for survival in a polluted environment.

Assessing immunotoxic effects is not straightforward, as the immune system is complex and multifaceted. This means that the chemicals can affect immunocompetence through a wide range of mechanisms and via diverse targets. Also, to establish the relevance of a toxicant-induced change in a selected immune parameter for the overall immune status of the organism is a difficult task, since the overall immunocompetence of the individual is more than the sum of the individual parts. It is the aim of the present communication to briefly discuss several aspects that can be of relevance in designing an immunotoxicological study. In particular, we will address which immune parameters might be selected at different levels of complexity, from simple indicative responses to the evaluation of altered immunocompetence, and how the response of immune parameters to chemical impact varies with the activation status of the immune system. For this discussion, we will focus on teleostean fishes, because (1) they are an important animal group in ecotoxicology, (2) knowledge on the fish immune system has impressively grown over the last decades what opens new avenues and possibilities to the study of immunotoxic effects, and (3) there exists a reasonably good body of both laboratory and field observations on immmunotoxic effects in this animal group. It is not the aim of this work to provide an comprehensive review on fish immunotoxicology, as there are several recent reviewss available (Rice 2001; Burnett 2005; Carlson and Zelikoff 2008), but to highlight specificities that have to be considered in assessing immunotoxicity.

The immune system of teleost fish — a starter

In general, the immune system protects organism homeostasis and integrity by monitoring any alteration of cells and tissues caused either by internal (age, neoplastic proliferation, etc.) or external (pathogens, chemicals, etc.) stressors.

The basic mechanism behind this is the recognition and discrimination between self and non-self. The immune system protects organism integrity, it ensures appropriate function of organs and tissues against invading pathogens, and thus, is critical for survival and fitness of the organism. General design principles of immune systems include (1) combination of general and specific responses; (2) division of tasks among specific immune cell populations, both resident and migratory ones; (3) intensive communication and signaling among the various immune system components; (4) a balancing of forces, e.g., between pro- and anti-inflammatory signals; and (5) extensive variability and continuous innovation to be able to cope with antigenic diversity, for instance, by polymorphism and polygeny (Trowsdale and Parham 2004). In addition, the immune system must be in a state of preparedness even in the absence of any antigenic challenge, it must be in strategic locations within the organism in order to sense and communicate information on invading foreign material, and it must be able to rapidly replenish immune cells.

The immune system of teleost fishes can be subdivided broadly into the following categories (Fig. 1) which differ in the speed and specificity of response (Rice 2001; Burnett 2005; Carlson and Zelikoff 2008). A first line of defense is represented by external barriers separating the fish from its environment, i.e., the epithelia of skin, gills and alimentary canal. These epithelia work as mechanical barriers to invading pathogens, but they also contain humoral (anti-bacterial peptides, complement factors, antibodies, etc.) and cellular (immune cells) immune factors. A second category of immune defenses is the innate immune system which generates relatively rapid but non-specific response to invading pathogens. The innate immune system can be activated by pathogen-associated molecular patterns (PAMP) that are common to many pathogens, for instance, bacterial lipopolysaccharide (LPS). The counterpart to PAMP on the host side are pattern recognition receptors (PRR), which recognize either the foreign molecules or endogenous, host-derived alarm molecules (Magnadóttir 2006). Main effector elements of the innate immune system of fishes include humoral factors and cellular responses. Examples of the humoral factors are lysozyme, cytokines and chemokines, or the complement system which includes enzymes that promote inflammatory responses, assist in lysis of foreign cells, and stimulate the adaptive immune system. Cellular elements of the innate immune system include phagocytic cells like granulocytes, monocytes and macrophages, or natural killer cells. The main functions of these cells are to phagocytose tissue debris and microorganisms, to secrete immune regulatory factors and to bridge innate and adaptive immune responses. A third line of defense is the adaptive or acquired immune system, a set of humoral and cellular responses which are typically slower but pathogen-specific. Adaptive immunity provides organisms with a mechanism for deriving an almost limitless variation from very few genes (Litman et al. 2010), which represents a major advantage in the fight against genetically variable pathogens. Cells involved in the specific immune system are T- and B-lymphocytes which mediate the cellular and humoral responses, respectively. The lymphocytes possess antigen-specific receptors that are activated by antigenic peptides bound to Major Histocompatibility Complex (MHC) proteins that are displayed by either infected host cells (MHC Class I) or by professional antigen-presenting

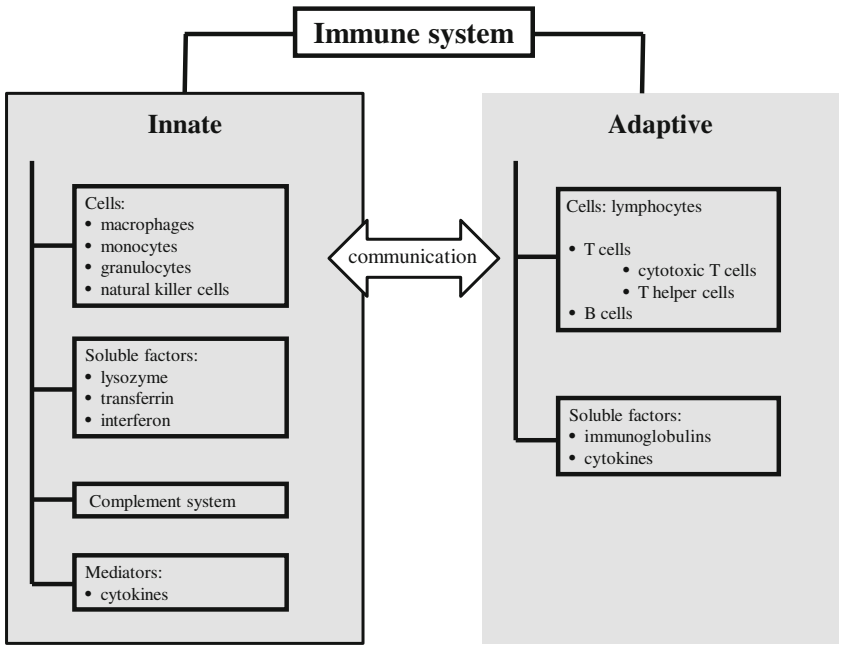

Fig. 1 Schematic and simplified overview of key elements of the immune system of teleost fish

cells (MHC Class II). Although the characterization of piscine T-lymphocytes is by far not as progressed as in mammals, it is clear that fish possess both antigen-presenting T-helper cells (CD4-like) and cytotoxic T-cells (CD8-like) (Fischer et al. 2006). Fish B-lymphocytes produce immunoglobulins which are primarily tetrameric IgMs (Warr 1983), instead of the pentameric immunoglobulins of mammals.

The tissues of the immune system are referred to as lymphoid organs. Unlike mammals, teleost fish lack hematopoietic bone marrow and identifiably lymph nodes, but the primary site of hematopoesis in teleosts is the head kidney which phylogenetically and ontogenetically represents the pronephros. Further immune organs and tissues of fish include thymus, spleen, gut-associated lymphoid tissue (GALT) and interstitial tissue in the excretory kidney. The morphology of fish lymphoid tissues has been reviewed previously (Zapata et al. 1996; Press and Evensen 1999; Carlson and Zelikoff 2008).

The immune system of fishes is often considered to be a primitive one. This notion may be related to the fact that fish do not separate the tissues for generation of myeloid and lymphoid immune cells, as do mammals. Also, the adaptive immune system may be less functional than in "higher" vertebrates, as it evolves only within the group of fishes (Litman et al. 2010). Irrespective of how correct the view of a "primitive" immune system of fish is, this system is apparently efficient enough to support ecological success of this group of vertebrates against a plethora of infectious pathogens and this within the aquatic environment which is highly supportive to pathogen transmission and infection.

Assessing immunotoxicity

The immune system as a fully dispersed system being present in most tissues and organs is readily accessible to toxicants. Irrespective of the toxicant uptake route, be it via respiratory epithelia and skin, or be it via the gut, immune system components will be exposed. They are exposed again during the distribution of chemicals within the body fluids, i.e., blood and lymph vessels. Finally, immune system components are present within key tissues of chemical toxicity and metabolism such as liver or kidney. Also, the lymphoid tissues themselves are important sites of chemical toxicity and metabolism (Carlson et al. 2004; Nakayama et al. 2008a; Valdez Domingos et al. 2011).

Immunotoxic chemicals have been shown to cause adverse health effects either by suppressing the immune capacity of exposed organisms, or by inappropriate stimulation of the immune system, for instance by changing duration or specificity of immune responses (Burns et al. 1996). Due to the network nature of the immune system, toxic responses can be rather diversified, for instance, exposure of mammals to 2,3,6,7-tetrachlorodibenzo-*p*-dioxin can result in compromised immunocompetence (Smialowicz et al. 2004), increased risk of autoimmunity (Mustafa et al. 2008) and dysregulated inflammation (Luebke et al. 2002). A particular risk associated with immunotoxicants is that early life exposure may result in persistent, lifelong modulation of immune capacity. This is well documented for mammals (Dietert 2009; Winans et al. 2011), but also for fish there are indications that developmental exposure can lead to persistent immune dysfunction (Ottinger and Kaattari 2000; Milston et al. 2003, Segner 2011b).

Given the complexity and multifaceted nature of the immune system, a single assay or parameter is rarely sufficient to assess immunotoxic effects. Instead, to gain an appropriate representation of toxicant effects on an individual's immunocompetence, it needs a range of techniques and endpoints. The critical questions are (1) which immune parameters out of the many possible parameters to select, and (2) how to evaluate the significance of the selected parameters for the overall immunocompetence of the organism? For the field of human toxicology, frameworks and regulatory guidelines for immunotoxicity risk assessment of chemicals are established, including structured approaches to the assessment of immunostimulation, immunosuppression, sensitization and allergic response, and autoimmunity (Burns et al. 1996; Hinton 2000; Schulte and Ruehl-Fehlert 2006). The World Health Organization (WHO) is currently developing a comprehensive "Guidance for Immunotoxic Risk Assessment of Chemicals" which summarizes a wide range of assays and the information that can be derived from these assays. Mammalian immunotoxicologists have organized these tests in tier approaches. Tier I provides screening for general immunomodulating effects, including, for instance, hematological parameters or lymphoid organ weights, but also including simple assays of cell-mediated defences or the plaque forming cell (PFC) assay to measure antibody-based humoral immunity. Tier II provides a more comprehensive assessment including cell surface marker analysis, assays of cytotoxic T-cell functioning, or host resistance models (Burns et al. 1996; Hinton 2000; Schulte and Ruehl-Fehlert 2006). No comparable framework is available for the ecotoxicological risk assessment of chemicals. Existing regulations such as REACH in Europe do not require the evaluation of immunotoxicity — although this may change with the increasing attention to specifically acting environmental contaminants such as pharmaceuticals, with many of them, e.g., diclofenac, being actually designed as immunomodulators. In contrast to predictive risk assessment, ecotoxicological field and monitoring studies frequently employed immune parameters (e.g., Rice et al. 1996; Luebke et al. 1997; Galloway and Depledge 2001; Garrigues et al. 2001; Schmidt et al. 2003; Skouras et al.

2003). Widely used immunotoxicity assays with both vertebrates and invertebrates are, for instance, determination of respiratory burst and phagocytotic activity of immune cells, or the inhibition of immune cell proliferation (e.g., Zeeman and Brindley 1981; Bols et al. 2001; Galloway and Depledge 2001; Hutchinson et al. 2003). Currently, the selection of the immunotoxicological methods used in ecotoxicology still largely depends on the availability of well-established, standardized assay protocols, whereas a comprehensive, tiered testing strategy has not yet been developed. The following discussion organizes immunotoxicity methods available for fish in five sections, which represent different levels of complexity and immune system integration, and a tiered immunotesting strategy with fish might follow these levels: immune mediators, humoral immune effectors, cellular immune defenses, macroscopical and microscopical responses of lymphoid tissues and organs, and host resistance.

Assessing fish immunotoxicity: immune mediators

During an immune response, the diverse and dispersed immune cells have to communicate in order to mount a coordinated action. The connection between the immune cells as well as with non-immune cells is achieved by a vast network of soluble mediators, the cytokines. Nearly all immune cells secrete cytokines that may have local or systemic effects, and they have functions in both innate and adaptive immunity. Examples of cytokines are the interleukins, tumor necrosis factors and transforming growth factors. Measurement of immune mediators in immunotoxicity studies has been strongly promoted and made easy through the emergence of molecular techniques including reverse transcription polymerase chain reaction (RT-PCR) or transcriptomic arrays (e.g., Harms et al. 2000; Koskinen et al. 2004; Quabius et al. 2005; Eder et al. 2008; Nakayama et al. 2008b; Jin et al. 2010; Jovanovic et al. 2011). These techniques provided gene sequences for many piscine cytokines (Saeij et al. 2003; Goetz et al. 2004; Secombes and Cunningham 2004), and they enable rapid screening of chemical effects on expression of immune mediators. They also may provide insight into the mechanisms of immunotoxic actions of chemicals, particularly when utilizing multigene expression tools such as microarray platforms which connect single gene changes to the response of whole pathways and physiological functions (Koskinen et al. 2004; Nakayama et al. 2008b). The main limitation of using immune mediators as endpoints in immunotoxicity assessment comes from the functional interpretation — what actually are the consequences of a change in a particular cytokine for the overall immune status of the animal? While an answer to this question may be possible for mammals, where the role of individual cytokines is fairly well understood, this is difficult for fish where the knowledge of the fish immune system and its responses under stress is still too limited to understand the toxicological implications of down- or up-regulation of a specific immune mediator, at least as long as this is a stand-alone measure. Analysis of immune mediators can be of value, however, if linked, either correlationally or mechanistically, to functional immune endpoints in order to get insight into the physiological or pathological consequences of the altered expression of the mediators.

Assessing fish immunotoxicity: humoral effectors

The fish immune system possesses a number of soluble humoral factors that have the function to cross-react with or attack foreign antigens. Exposure of fish to toxic chemicals is able to modulate concentrations and synthesis of the humoral factors. In the adaptive immune system, the key humoral effectors are the pathogen-specific antibodies; in the innate immune system, humoral factors include complement, acute phase proteins, lysozyme or interferons (Yano 1996). These factors are found in the serum, mucus and eggs of fish; they recognize molecular motives shared by a wide variety of pathogenic microorganisms. Interferons are key effectors in the host response against viral pathogens. Lysozyme is an enzyme that disrupts the cell walls of bacteria by splitting glycosylic linkages in the peptidoglycan layers. Acute phase proteins are plasma proteins that are synthesized in the liver in response to tissue damage, infection or inflammation. They function in a variety of activities including repair of tissue damage, killing of pathogens, limiting pathogen dispersal or inactivation of proteases. Examples of acute phase proteins in fish are C-reactive proteins, transferrins, α-macroglobulin and anti-microbial peptides (Bayne and Gerwick 2001).

Effects on soluble humoral factors of fish have been shown for a wide variety of chemicals (Zeeman and Brindley 1981; Bols et al. 2001). This applies for humoral factors of the innate immune system (e.g., Bols et al. 2001; Prabakaran et al. 2006; Fatima et al. 2007; Kreutz et al. 2011) as well as of the adaptive immune system (e.g., Zeeman and Brindley 1981; Albergoni and Viola 1995). While responses of the adaptive immune system are usually assessed through measuring plasma immunoglobulin (Ig) levels (Siwicki and Anderson 1993; Dautremepuits et al. 2004), several tests are available for soluble factors of the innate immune system. For instance, the anti-microbial activity of serum is easily tested by applying different dilutions of serum to live bacteria suspensions and determining the viability of bacteria by the spread plate method (e.g., Arkoosh et al. 2005). Lysozyme activities can be assessed by adding test sera to defined bacterial cell suspensions, and

then measuring the lysis of the bacterial cells (Alexander and Ingram 1992). Such assays, which offer the advantages of low costs and easy application, are widespread and partly commercially available as kits. Another commonly used assay parameter is the complement activity of sera. The complement system consists of a series of proteins which are involved in pathogen cell lysis, in clearance of pathogen-infected host cells and in the formation of chemotactic peptides to attract immune cells. In teleost fish, the complement system has been found to possess components of the classical, alternative, and lectin pathways, which represent enzymatic cascades that convert inactive precursor proteins into active ones (Nonaka and Smith 2000). Teleosts possess a more diverse array of complement components than other vertebrates, for instance, multiple C3 forms have been described (Sunyer et al. 1996). Serum complement activity in fish is usually tested by measuring haemolytic activity using sheep red blood cells count (Yano 1993), plague count assays (e.g., Prabakaran et al. 2006) or similar assays. Another possibility is to determine the bacteriolytic activity of fish complement by using recombinant bacteria which express fluorescent or luminescent marker genes. Dilution series of trout serum were combined with bacteria suspension and then assessed for total complement (TC) activity and alternative complement pathway (ACP) activity by measuring the luminescence of the solution. These complement assay are commonly used due to their low costs, easy handling and quick results production. The caveats, however, are unspecific responses when measuring TC activity, bias of results due to a) temperature dependence of the complement system response and b) different binding and affinity properties of the various complement components isoforms. Also, inaccurate sample preparation can influence the test results.

Fish immunotoxicity: cellular defenses

Immune cells mediate four key functions in the defense of the organisms against pathogens: (1) to attack, lyse and/or phagocytose the pathogen; (2) to attack, lyse and/or phagocytose pathogen-infected or malignantly altered host cells; (3) to produce soluble humoral factors; and (4) to transfer information, for instance, antigen presentation. It is clear that chemical interference with these functions can easily translate into altered immunocompetence of the host. Therefore, assays measuring changes in immune cell numbers, composition, proliferation or function play a central role in immunotoxicity studies.

In fish, characterization and classification of the various immune cell types has been hampered for long, on the one hand because of pronounced morphological variation of the cells both within and between species, and, on the other hand, due to the lack of defined molecular and cellular markers for the individual cell types (Carlson and Zelikoff 2008). However, over the last decade, substantial progress has been achieved in the existing knowledge on immune cell types of teleost fish (e.g., Shen et al. 2002; Castro et al. 2011). The innate immune system of fish includes two general cell types, i.e., phagocytic cells (monocytes/macrophages, granulocytes) which attack invading pathogens, and natural killer cells or nonspecific cytotoxic cells which initiate killing of infected cells. However, in contrast to cytotoxic T-cells, they do not require specific antigen presentation (Rice 2001). The functional analysis of phagocyte activities under chemical exposure is commonly achieved by determining respiratory burst activity or phagocytosis activity (e.g., Rice et al. 1996; Zelikoff et al. 2000; Bols et al. 2001). The respiratory burst of phagocytes involves reduction of oxygen to the anionic superoxide radical, which may subsequently undergo conversion to other reactive oxygen species (ROS). Together, the ROS produced during the respiratory burst are used as potent antimicrobial agents. Methods to measure respiratory burst include, for instance, reduction of the dye nitro blue tetrazolium, or chemoluminescence analysis (Köllner et al. 2002). The phagocytosis assay measures the effect of toxic chemicals on the ability of phagocytic cells to ingest foreign material. To this end, the phagocytic cells are isolated and confronted ex vivo with a labeled antigen or with bacteria, subsequently, the number of phagocytosing cells or the number of particles engulfed per cell is measured, for instance, by observation of the cells under the microscope or by flow cytometry (Harford et al. 2006; Nakayama et al. 2007).

The cellular component of the adaptive immune system of fish is based on the lymphocytes, involving B-cells being responsible for the antibody-mediated humoral immunity, and the T-cells being responsible for the cell-mediated immunity. The T-cells of fish are composed of several subpopulations which are getting increasingly better characterized (e.g., Castro et al. 2011; Chang et al. 2011). Chemical effects on T- and B-cells of fish are often estimated from lymphocyte proliferation assays (Zelikoff et al. 2000; Carlson et al. 2002; Iwanowicz et al. 2009). These assays evaluate if the functional capacity of lymphocytes to multiply in response to a pathogen is impaired under exposure to a toxicant. To this end, lymphocytes are harvested, for instance, from the spleen, and are stimulated in vitro with potent mitogens such as conconavalin A or LPS. After several days of incubation, cell proliferation is measured by incorporation of, e.g., [^{3}H]thymidine, or other indicators of cell proliferation (Carlson et al. 2002; Köllner et al. 2002). The comparison of control and exposed cells reveals if the chemical exposure led to an altered proliferative capacity of the lymphocytes. Another possibility to test for

chemical effects on T-lymphocytes is to measure the specific cell-mediated cytotoxicity of lymphocytes isolated from control or pollutant-exposed fish (Fischer et al. 2006). In contrast to the assay on cell-mediated cytotoxicity, the plaque-forming cell assay has been used as an indicator of the chemical's effect on the ability of the exposed fish to mount a humoral antibody response (Arkoosh et al. 1991; Carlson et al. 2002; Prabakaran et al. 2006). For this assay, control and exposed fish are primed with a foreign antigen, for instance, sheep red blood cells (SRBC). Some time after the SRBC injection, spleen cells are isolated from control and exposed fish, mixed with SRBC and complement, and plated onto Petri dishes. B-cells from the spleen then secrete anti-SRBC antibodies, which, together with the complement, lead to SRBC haemolysis. The resulting plaques can be measured to reveal if B-cells from the chemically treated fish show an altered capability to generate an antibody response.

The aforementioned assays are ex vivo assays, i.e., the fishes are treated in vivo with the toxic chemical, immune cells are then isolated and their functionality is tested outside the fish by measuring a cell-specific response such as ability for phagocytosis or the ability for antibody secretion. However, in order to learn which immune cell types are targeted by the toxic chemicals and how this compromises the immunocompetence of the exposed organisms, it is important to assess also chemical effects on immune cell number, composition of the immune cell population as well as their distribution and dynamics in the living fish. Technically, this can be achieved by means of flow cytometry of immune cells, either from the blood ("peripheral blood leukocytes") or from lymphoid organs. The challenge in this approach is to identify the individual immune cell types. Flow cytometric analysis enables to discriminate cell types by size and granularity (Evans et al. 1987; Moritomo et al. 2003), but the exact identification of the specific immune cell types, needs additional tools such as analysis of cell type-specific marker genes or surface marker-directed antibodies. The limitation here is that currently only few marker antibodies for immune cell types of fish species do exist.

Chemical effects on fish immune cells are studied not only in vivo or ex vivo, but also in vitro, both on established cell lines and on primary isolates (Bols et al. 2001; Quabius et al. 2005). Probably the best application fields for in vitro studies with isolated fish immune cells are mechanism-oriented studies, for instance, which immune cell types are targets of chemical toxicity due to the expression of xenobiotic receptors, or how do chemicals influence signaling pathways in the immune cells. The isolation of the immune cells is usually performed from the head kidney, the spleen, or the blood. The advantage and also disadvantage of isolates from tissues is that they contain not only mature but also all developing stages of immune cells. This makes an exact cell type definition difficult. A rough fractionation can be achieved by means of density gradient centrifugation after isolation (Garduno and Kay 1994), and by subsequent monolayer cultivation, what will lead to a preferential attachment of phagocytic/macrophage cells, while the lymphocytes will not attach and can then be washed away (MacKenzie et al. 2006).

Two caveats should be kept in mind when doing in vitro chemical exposures with isolated immune cells. First, the immune response in vivo is an integrated response, based on networking and communication among the numerous immune system components. This characteristic element is absent in vitro, what means that the relevance of in vitro exposure experiments has to be interpreted cautiously. Second, exposure concentrations used in the in vitro experiment should remain well below cytotoxic concentrations, in order to not confuse cytotoxicity with immunotoxicity.

Fish immunotoxicity: organ and tissue responses

Changes in organ size, organ histology or in blood composition are integrative endpoints for assessing immunotoxicity, whereas their diagnostic value for establishing cause–effect relationships is limited. Particularly for field studies with fish, however, these parameters may be of value. Changes in relative organ size, for instance, splenosomatic index, as well as changes of hematological parameters such as white blood cell count or haematocrit, can be easily measured and have been successfully employed in fish biomonitoring programmes (Faller et al. 2003; Skouras et al. 2003). Processing and evaluation of histological samples is labour-wise more demanding, but sampling under field conditions is easy. The value of histopathological studies is that they provide information on the targets of immunotoxic chemicals, the relation to organ damage and adverse outcome, and what the underlying modes of action could be (Grinwis et al. 2000; Hoeger et al. 2005). Naturally, all these techniques have their value also for laboratory immunotoxicity testing. In particular, histopathology is a routine tool in mammalian immunotoxicity testing, which provides a reasonable level of accuracy in immunotoxicity screening, although it is apparently less sensitive than functional parameters (Germolec et al. 2004). In immunotoxicological studies with fish, histopathology is surprisingly little used. It is an interesting question to what extent the limited use of histopathology in fish immunotoxicology is reflective of a low information value of this technique for immunotoxicological purposes, or if it reflects a more technical problem, i.e., the shortage of trained fish histopathologists being able to perform this type of analyses (Spitsbergen et al. 2009).

Fish immunotoxicity: host resistance

Host resistance assays inform on whether chemical exposure impairs the ability of the fish host to resist to infection by foreign antigens. As it is the function of the immune system to defend the host against pathogens, demonstrating that this function is compromised by chemical exposure is the ultimate proof of immunotoxicity. Host resistance assays are performed as challenge assays in which the fishes are exposed to a defined concentration of a virulent pathogen, and then the cumulative mortality is determined The pathogen can be administered by bath immersion, through the oral route, or by injection. The possible influence of chemicals on the host resistance is estimated from the comparison of the cumulative mortalities in control and toxicant-exposed fish. Chemical exposure usually takes place before or parallel to the pathogen challenge (Köllner et al. 2002; Wenger et al. 2011), but it also can occur after pathogen infection of the fish. An example to the latter approach is provided by the study of Song et al. (2011) who exposed virus-infected Japanese flounder, *Paralichthys oliveaceus,* to heavy oil, and observed that fish exposed to both stressors suffered significantly higher mortalities than fish exposed to only heavy oil or only virus. A caveat to host resistance assays is that they represent a partly artificial situation and that the outcome can vary with factors such as the administration route or administration method of the pathogen. Also, the endpoint in this assay — mortality — does not inform on the underlying processes; strictly speaking, it does not prove that a chemically induced elevation of mortality is due to a immunotoxic action of the chemical. Thus, it is advisable to restrict effect analysis in the challenge assay not only to mortality but to include additional endpoints from the panel of methods described above.

A particular strength of the host resistance assay is that it reveals the action of the toxic chemical upon the activated instead of the resting immune system (Köllner et al. 2002). As the physiological function of the immune system is the defense against invading pathogens, it is primarily the chemical impact on the immune system in its active form what matters, and not so much the chemical impact on the resting system. If a chemical affects a certain immune parameter when the system is in the "stand-by mode", this is not necessarily a negative effect. However, if the chemical impairs the ability of the host to mount an effective immune response to the invading pathogen, this clearly has adverse consequences. Köllner et al. (2002), therefore, emphasized that assessment of immunotoxicity should be based on measuring chemical effects on the activated rather than on the resting immune system. This applies for immune parameters measured in vivo, but it also applies for ex vivo measurements, for instance, the lymphocyte proliferation assay (see above) would be done with cells isolated from fish exposed both to chemicals and pathogens while controls would be sampled from fish infected with pathogens but not exposed to toxicants.

The fact that the immune response to an environmental compound may differ between the resting and the activated system may be illustrated by the study of Wenger et al. (2011) on the impact of exogenous estrogens on the immunocompetence of rainbow trout: juvenile rainbow trout were exposed for 4 weeks to 17beta-estradiol (E2), as prototypic "endocrine disruptor. The E2 concentrations were sufficiently high to induce an estrogenic response such as the induction of the estrogen biomarker, vitellogenin, whereas concurrent effects on the immune system — as assessed by analysing the complement system — were not detectable. At this stage of the investigation, the conclusion would have been that estrogenic exposure remains without effect on the immune status of trout. However, when the fishes were challenged with the bacterial pathogen, *Yersinia ruckeri*, a significant difference between the control and the estrogen-exposed groups became evident: The estrogen-treated fishes were not able to up-regulate the expression of key complement genes as a defence against the infectious pathogens, whereas the control fishes well up-regulated their complement gene expression. In line with this, the estrogen groups suffered significantly higher mortalities than the control groups. Apparently, the estrogenic treatment had an impact on immunocompetence of the trout, but this impact was visible only in the activated immune system.

Conclusions

From the discussion above, it appears that there exist quite a number of assays to detect local and systemic immunotoxic effects, against the inherent complexity of the immune system. This applies not only for fishes, which were considered here, but also for other vertebrates as well as for invertebrate wildlife species (cf. Demas et al. 2011; Pedersen and Babayan 2011). Over the past years, the methodologies for assessing fish immune parameters have grown substantially, and it is expected that they will grow even more in the future, due to the steadily growing knowledge on non-mammalian immune systems and with the rapid progress of genomic and post-genomic technologies. The limitations to an improved consideration of immunotoxicity in ecotoxicological risk assessment are thus not coming from technological constraints, but the challenges are of different nature:

– Missed aspects of immunotoxicity: Immunotoxicological studies in ecotoxicology have focused almost exclusively on immunosuppressive effects of toxic chemicals. From mammalian immunotoxicology, we know about the importance of other toxicant-induced immunological

disorders, such as autoimmunity and hypersensitivity. The question arises if this type of immunological responses to toxic exposure is without relevance outside the mammalian world, or if we have simply missed them up to now (cf. Rice 2001). Also, the possible impact of long-term, low-dose exposure to specifically acting compounds such as endocrine disruptors and pharmaceuticals on wildlife immunocompetence has not yet received appropriate attention (Rice 2001; Hoeger et al. 2005; Thilagam et al. 2009; Casanova-Nakayama et al. 2011).

– Diversity of immunity: In immunotoxicology, we have to deal not only with the complexity of the immune system, but also with its diversity within and across species (Pedersen and Babayan 2011). Within populations, individual differences of the immune capacity arise from physiological (age, sex, reproductive state, etc.) and genetic differences. This — evolutionary advantageous — variability affects host susceptibility and fitness (Lazarro and Little 2009), and it affects the consequences of chemical exposure on host immunity. Such variability is also of relevance for the extrapolation of laboratory immunological findings to the field, in particular as laboratory studies — for the sake of reduced variability — often use genetically fairly homogenous strains (Demas et al. 2011; Pedersen and Babayan 2011). Apart from the fact that individual variation of immune parameters gives rise to high standard deviations of experimental results — what sometimes makes immunotoxicological studies difficult to defend against reviewers — we currently lack understanding what this variation means with respect to the consequences of immunotoxic effects for the populations. These problems are even more pronounced if it comes to inter-species variation of immune properties and responses. It is clear that we will not be able to answer these questions from a purely toxicological viewpoint, but here we need integration with areas such as ecoimmunology, which places the immune response into the ecological and evolutionary contexts of species (Segner et al. 2011).
– Integration into ecotoxicological risk assessment: As addressed above, adverse consequences of exposure to immunotoxic chemicals for the host may become visible only under co-exposure to pathogens (Arkoosh et al. 1998; Springman et al. 2005; Song et al. 2008; Wenger et al. 2011). For predictive hazard testing of chemicals this could mean that a chemical concentration that by itself has no measurable adverse effect in a standard toxicity test, and thus would be considered as "no effect concentration", may still have an adverse effect on the organism by enhancing its vulnerability towards pathogens (Springman et al. 2005). With respect to the field situation, this would mean that a chemical being present at concentrations below the predicted no-effect concentration — and, thus, inducing per se no adverse effect — still can get adverse by increasing host susceptibility to pathogens. The crucial point is that we deal here with indirect effects arising from exposure to multiple stressors what makes it difficult to establish cause–effect relationships (Galloway and Depledge 2001). Clearly, we need more conceptual understanding of combination effects of chemicals and pathogens (Springman et al. 2005; Spromberg and Mador 2005), but approaches for the assessment of multiple stressor scenarios in ecotoxicology are emerging only now.

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