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Improving disease prevention and treatment in controlled fish culture

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Abstract. The aim of the work was to evaluate long-term results of studies focusing on improving methods for preventing and treating fish diseases using selected natural and syntetic immunomodulators and vaccines in fish culture. Simultaneously, attention is drawn to infectious or environmental threats against which appropriately composed immunoprophylaxis can be used in production cycles. Fish culture is intensifying in Poland and globally, which means that the role of prevention and well-designed prophylaxis is of increasing significance to the prevention and treatment of fish diseases. Currently, 33 fish species are cultured in Poland as stocking material or for production. The primary methods for preventing diseases in controlled fish culture are ensuring the welfare of fish and other prophylactic methods, including immunoprophylaxis. Many infectious and non-infectious threats that can cause direct losses and limit fish culture are present in the aquatic environment. Fish diseases generally stem from the simultaneous action of many factors that coincide and are difficult to distinguish. Pesticides (organochlorine insecticides, organophosphorus herbicides), aromatic hydrocarbons, pentachlorophenol, heavy metals, and chemotherapeutics are particularly toxic to fish. Biodegradation, which is continual in aquatic environments, is a process by which toxic and other substances that negatively affect fish become bioavailable and impact the immune system, the functioning of which is a specific bioindicator of environmental quality. Innate immunity plays a key role in the defense against disadvantageous factors, which also include pathogens. Immunomodulation methods can protect resistance mechanisms, thereby increasing disease prevention and treatment in controlled fish culture.

Keywords: innate and adaptive immunity, fish diseases, immunoprophylaxis, immunomodulation

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Introduction

The development of aquaculture has led to growing interest in the prevention and treatment of fish diseases. The primary aim of improving rearing methods is to ensure fish welfare through modern technological solutions that permit achieving maximum production results. Preventative and prophylactic methods permit minimizing negative impacts stemming from threats to fish health. Implementing immunomodulators into culture practice is especially significant since they can stimulate the immune system. The foundation for maintaining physiological balance and homeostasis in fish is the proper functioning of innate, or natural, and adaptive, or acquired, resistance mechanisms. Simultaneously, the efficiency of their functioning is a sensitive bioindicator of culture and environmental conditions. This presents certain limitations especially in the environment of intensive aquaculture since large stocks are kept at high densities, thus

generating permanently stressful conditions that threaten fish welfare.

Continued growth in production in this sector can be expected in the future. Globally, the importance of aquaculture is continually on the rise, and the indicator of mean annual growth in the 2003-2013 period was 6.2% (FAO 2014). The inland aquaculture sector in Poland is based on, above all, the culture of two species of freshwater fish – carp, *Cyprinus carpio* L. (50.6% of total production in 2014) and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (40.1% of total production in 2014). The estimated value of sales of aquaculture production earmarked for consumption in Poland in 2014 alone was 369.9 million PLN, which was higher by 54.8 million PLN (17.4%) in comparison to that in 2013 (Lirski and Myszkowski 2015). Growth in production in this agricultural sector has been facilitated by the dynamic development of biotechnologies for the artificial spawning and culture of fish. Progress in this field has been possible thanks to the introduction of many new species of fish, i.e., wels catfish, *Silurus glanis* L.; African catfish, *Clarias gariepinus* (Burchell); various sturgeon species and its hybrids; perch, *Perca fluviatilis* L., whitefish, *Coregonus lavaretus* (L.), pikeperch, *Sander lucioperca* (L.), pike *Esox lucius* L.; European eel, *Anguilla anguilla* (L.); sea trout, *Salmo trutta* L.; salmon, *Salmo salar* L.; grayling, *Thymallus thymallus* (L.) (Kujawa et al. 2006, Robak 2006, Robak and Przystawik 2007, Robak et al. 2007, Grudniewska et al. 2009, 2012b, Szczepkowski 2011, Ulikowski 2011, Kozłowski et al. 2012, Kolman 2015, Zakęś and Rożyński 2015). Currently, rearing and stocking material in Poland is produced from 33 fish species and in 2014 estimated production was 11,596 tons, which represents an increase of 16% in comparison to 2013 (Lirski and Myszkowski 2015). Aquaculture also plays an important role as a tool of active biodiversity conservation in aquatic ecosystems in many regions of Europe, including in the Baltic Sea basin (Robak and Przystawik 2007, Mickiewicz et al. 2011, Kolman 2015). However, one of the principle aims of production is to meet the increasing consumption demands of both humans and animals. This creates an additional challenge for fish producers as well as for veterinary doctors and ichthyologists. New species that are undergoing the process of adapting to controlled conditions often develop health problems that have yet to be diagnosed and that are often caused by factors in their immediate environments (Bergmann et al. 2006, Johansen et al. 2011, Terech-Majewska et al. 2011, 2015b, Bernad et al. 2016a). Identifying the requirements of a species in new rearing conditions requires time and additional diagnostic and therapeutic methods. Currently, innate and adaptive immunoprophylaxis is becoming increasingly important in this sector, and for species that are new to aquaculture. The significance of immunoprophylaxis will increase, especially with regard to those species that are in consumer demand, but, because they have not yet been fully domesticated, they are not yet ready for culture under controlled conditions. These include, for example, common whitefish, perch, pikeperch, and pike. Then being reared as restocking material in closed systems (RAS), these species often have to be prepared appropriately immunologically to allow them to better adapt to differing environmental conditions.

Immunomodulation can be included at various stages of fish development beginning with spawners (in the pre-spawning period), in progeny from the moment they begin feeding exogenously, and also each time fish are subjected to technological procedures (either before or after) and in other instances that generate stress in fish (Ingram 1980, Siwicki et al. 1995, 1998a, Anderson and Siwicki 1996, Almendras 2001, Kazuń and Siwicki 2005, Bowden et al. 2007, Grudniewska et al. 2010). Well-designed immunomodulation programs permit protecting of immune system function before it is compromised by manipulation stress or the environment. However, the most effective fish disease prevention is adaptive immunoprophylaxis based on vaccinations that are chosen according to the needs of culture facilities and targeted prevention programs. Using auto-vaccines that are prepared from bacterial strains isolated from the fish, the facility, a river basin, or even an entire region, appears to be a particularly appropriate prevention method (Siwicki et al.

2001a, 2004a, 2010a, 2010b, Siwicki and Szweda 2010, Kozińska and Pękala 2012). Highly effective prophylaxis is noted with microorganisms that are conditionally pathogenic, i.e., *Aeromonas* spp., *Yersinia ruckeri*, and *Pseudomonas* spp. In aquaculture, new rearing technologies and the negative impacts of xenobiotics, as well as climate changes, facilitate the emergence of new pathogens, and these factors can also alter the pathogenic profiles of well-known diseases that are partially controllable. Traditional prophylactic methods, including supplying high quality feed that guarantees good fish condition and immunity in developed feeding programs, on-going disinfection, or periodic metaphylaxis with antibiotics or sulfonamides, remain the foundation of preventing and treating diseases of fish in culture facilities (Wedemeyer et al. 1978, Terech-Majewska et al. 2004a, 2010a, 2014b, Grudniewska et al. 2006, 2014, Kowalska et al. 2006, Szczepkowski et al. 2008, Własow and Guziur 2008, Pękala et al. 2015b). It is likely, however, that using chemotherapeutics will be considered as a final alternative when other methods fail. The increasing resistance of microorganisms to antibiotics raises questions about the legitymacy and the cost effectiveness of using antibiotics in aquaculture. Additionally, it is known that antibiotics used in aquaculture can have an immunosuppressive effect, e.g., oxytetracycline, norfloxacin, ciprofloxacin, florfenicol. *In vitro* and *in vivo* studies both confirm statement (Sierosławska et al. 2000, 2005, 2007, Terech-Majewska et al. 2006). Usage of antibiotics has to be limited due to their harmful impact on the environment and difficult natural biodegradation (Samuelsen et al. 1992, Harnisz 2013, Gothwal and Shashidhar 2015, Harnisz et al. 2015). Their use is also limited by the results of studies that monitor prohibited substances in the tissues of animals for consumption, including fish (Szkucik and Maćkowiak-Dryka 2013). This is why work is being done to render antibiotic therapy a method that will only be used as an intervention to limit immediate losses. This can be achieved through the application of immunomodulation methods that can be incorporated in each stage of culture to provide protection with regard to biological, physical, and chemical threats (Siwicki et al. 1994a, 1998f, 2006a, 2011c, Terech-Majewska et al. 2004c, 2014c, 2016a, Singh et al. 2010). Significant threats to fish are found in aquatic environments and include toxins, pesticides, heavy metals, and petroleum compounds, all of which can impair fish immunity and predispose them to various health problems (Rymuszka and Siwicki 2004, Sierosławska and Rymuszka 2013). Methods for preventing and treating fish diseases are aimed mainly at controlling and limiting the occurrence of pathogens. Implementing such programs leads only to limiting their occurrence, but they are insufficient for completely eliminating them, which is why many diseases remain endemic despite long-term efforts to fight them (Matras et al. 2013, 2015).

Water is a particular environment for life in which all changes are subject to daily, seasonal, and climatic cycles. Despite monitoring, changing water parameters and the reactions of organisms cannot be fully regulated or predicted. The character of these changes determine culture possibilities as does the health status of fish. The immune systems of all animals are sensitive markers of all changes, and they can react to sublethal levels of xenobiotic compounds or their metabolites which, as foreign agents, have varied impacts on organisms. These agents can act as modulators (suppressive or stimulative) on cellular and humoral defense mechanisms and also on immune response. Cases in which the impact is negative can leave fish more susceptible to various diseases. The basic environmental factor that regulates the immune system and metabolism of fish is temperature at both the general and cellular levels. Other factors, such as light, insolation, oxygen, pH, which co-create environmental conditions, also have a significant impact (Sopińska 1992, Bowden et al. 2007, Bowden 2008). Using knowledge from immunotoxicology and diagnostics through to immunological markers and evaluations of environmental quality we can control the impact on the bodies of the fish, and in particular, on the innate defense mechanisms (Wester et al. 1994, Bly et al. 1997).

The aim of the work was to evaluate long-term results of studies focusing on improving the methods for preventing and treating fish diseases using selected natural and synthetic immunomodulators and vaccines in fish culture. Attention was focused on both infectious and environmental hazards against which immunoprophylaxis can be used in the production cycle.

Health hazards in fish culture

A range of both infectious and non-infectious hazards, that can cause direct losses and limit fish culture, are present in the aquatic environment. Fish diseases are generally the result of the simultaneous action of many, overlapping factors that are difficult to differentiate directly (Śnieszko 1974; Fig. 1). These factors can impact simultaneously environmental microflora and fish. The current system for the prevention and treatment of fish diseases reacts only after the appearance of new pathogens and their confirmed diagnosis, and creating effective legal procedures and the groundwork for their implementation is time consuming. Recently, the emergence of new, potentially pathogenic factors, which have not previously exhibited such activity, has been noted (Kozińska 2010, Noga 2010, Senger et al. 2012, Kozińska et al. 2013, Pękala et al. 2015b). Diseases that are especially hazardous are those that can cause epizootics, the control of which requires the involvement of many resources and services as well as action on an international scale (OIE 2016, Council Directive 2006, Regulation 2009). The prevention of diseases that are subjected to registration and monitoring is based on legal regulations that do not always keep pace with reality since the basis for diagnostics is known and implemented into the quality system of the diagnostic method (OIE 2003, 2009, 2016, Commission 2015). Methodologies applied in scientific research laboratories often outpace routine methods, which contributes to their development and improvement, and such laboratories often are the first to identify the emergence of potentially dangerous factors (Terech-Majewska et al. 2000, Siwicki et al. 2001e, 2006c, Bergmann et al. 2006, Grudniewska et al. 2011, Szarek et al. 2012, Robak et al. 2014). One example is the development of infectious hematopoietic necrosis (IHN) in salmonids (Table 1). The first case was confirmed in 2000, but

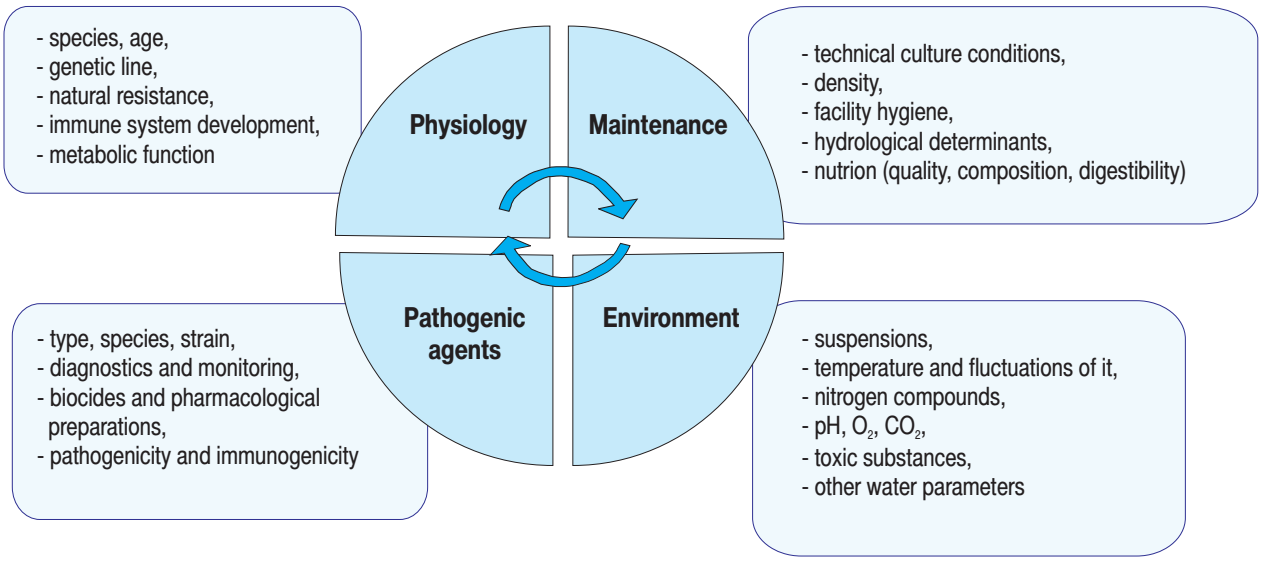

Figure 1. Determinants of fish diseases in controlled culture.

Table 1
Viral diseases of fish that must be reported according to the World Organization for Animal Health (l'*Office International des Épizooties* (OIE)) as required by EU law and which occur in Poland

Disease	Etiology	Susceptible species	OIE	EU (notification)	Poland (status)
Exotic					
Epizootic ulcerative syndrome – EUS	*Aphanomyces invadans*	fish of the genus Epalzeorhynchos	yes	yes	does not occur
Epizootic hematopoietic – EHN	EHNV	rainbow trout and perch	yes	yes	does not occur
Not exotic					
Infectious salmon anaemia – ISA	ISAV	Atlantic salmon, rainbow trout, sea trout, and others	yes	yes	does not occur
Koi herpesvirus infection – KHV	KHV/CyHV-3	carp and koi	yes	yes	occurs
Viral hemorrhagic septicemia – VHS	VHSV	rainbow trout, sea trout, grayling, Pacific salmon, pike, turbot, and others	yes	yes	occurs
Infectious hematopoietic necrosis – IHN	IHNV	rainbow trout, Chinook salmon, Atlantic salmon, and other anadromous fish	yes	yes	occurs
Infectious pancreatic necrosis – IPN	IPNV	salmonids	locally	no	no status
Spring viraemia of carp – SVC	SVCV	cyprinids	yes	no	no status

this study was not of an official character (Terech-Majewska et al. 2000). Almost simultaneously, the occurrence of the IHN virus (IHNV) in Poland was confirmed in the laboratory of the National Veterinary Research Institute in Puławy (PIWet) (Antychowicz et al. 2001). From the diagnosis of the first case of a disease or the confirmation of the occurrence of a pathogenic factor and the development of effective monitoring methods, and then prophylaxis frequently takes several years. With regard to the preceding example, official monitoring began in 2004, and by this time the disease had slowly spread throughout Poland.

Current problems pertaining to the health of cultured fish can be divided into two groups: those that are subjected to official veterinary monitoring and those that are left to be addressed by veterinarians in private practice and aquaculturists. Monitoring and official control in Poland and other EU countries concerns exotic diseases (those that do not occur in EU territory) and non-exotic diseases (those that occur in the countries of the EU) in accordance with the list of such diseases, which differs from that published by the World Organization for Animal Health (OIE, *l'Office International des Épizooties*) (Table 1). This system seeks to control the occurrence of infectious diseases that are important for protecting the health of animals. Fish disease epizootics are dynamic and varied depending on the month or the year (Terech-Majewska and Anusz 1996, Terech-Majewska et al. 2010b, Terech-Majewska and Siwicki 2010, Siwicki et al. 2011a). For example, during the period from January to June 2016 alone the viral hemorrhagic septicemia (VHS) virus (VHSV) was identified in two cases in the Pomeranian Voivodeship, while three cases of IHNV were noted, one each, in the Pomeranian, West Pomeranian, and Lower Silesian voivodeships. During the analyzed period, only one case infection of koi herpes virus (KHV) was detected in the Lublin Voivodeship. Monitoring salmonid viral diseases is done simultaneously for VHS and IHN and also for infectious pancreatic necrosis (IPN), the occurrence of which we learn of only through scientific publications (Matras

et al. 2013, 2015, Terech-Majewska and Siwicki 2013, Maj-Paluch and Reichert 2016). In many countries, despite no official requirements, the IPN virus, because of its characteristics, is monitored in some culture facilities, while in Norway it is monitored at all of them (Matras et al. 2015). A long-term program was conducted by the National Veterinary Research Institute in Puławy (PIWet) in 2014-2015 under the title "Analysis of epizootics occurring in Poland with regard to the most hazardous fish diseases: VHS, IHN, IPN, ISA, SDV, KHV, BKD," and within this framework fifty facilities were chosen for systematic sample collection simultaneously for a few viral diseases and for bacterial kidney disease (BKD). These studies confirmed the occurrence of the IPN virus at 10.5% of the analyzed facilities in 2014 and at 11% of them in 2015 (Matras et al. 2015). The VHSV virus has been detected in many European countries for many years, but despite long-term programs to fight it, its range of occurrence has not been diminished. In Poland monitoring in 2014 confirmed its occurrence at nine fish farm. The epizootic situation regarding the detection of the IHNV is similar; in 2014 three cases were confirmed. In Poland, there are 21 culture facilities that are free of the VHS and IHN viruses. Most frequently these facilities have the status of enclaves, which means that they are independent of the epizootic situation in the drainage basin, the results of monitoring studies conducted at them are negative, and they either have their own source of rearing material or they are supplied by facilities that are free of VHS and IHN. In Europe in 2012, however, only 17% of fish farms were free of VHS, while 20% of all facilities were free of IHN. In practice, this indicates that there is a need for official control that stems from the persistent epizootic situation. Controlling fish in the natural environment, where official studies are practically non-existent, is especially necessary.

In Poland another direction of VHS study is focused on common whitefish and pike as vector species. Hatching and rearing these fish species is conducted most frequently at salmonid farms using material that is obtained from the natural environment or from fry that is transferred for further rearing and restocking (Commission Regulation (EC) No 1251/2008 of 12 December 2008). As concerns the detection of other viral factors (e.g., AngHV-1 – anguillid herpesvirus 1, CCV – channel catfish virus) that are also hazardous to new species in aquaculture (eels), studies are of a scientific character and are generally realized within the frameworks of research projects (Davidse et al. 1999, Bergmann et al. 2006, Siwicki et al. 2006c, Kempter et al. 2014, Robak et al. 2014). The potential threat of AngHV-1 in open Polish waters is indicated by the results of the study by Kempter et al. (2014) who confirmed the presence of AngHV-1 genetic material in 50% of fish examined (14 ind.) from Lake Dąbie and in 28.6% (14 ind.) from the Szczecin Lagoon. Currently, the AngHV-1 virus has been identified in Germany, Greece, Holland, and France (Haenen et al. 2012). Studies conducted within the frameworks of long-term scientific projects have yet to detect the presence of these pathogens or viral genetic material in eel samples obtained from the Oder or Vistula basins (Robak et al. 2014). This signals the need to continue studies since eel is migratory which means that it could facilitate the spread of this virus in the natural environment and in culture facilities.

The actual detection and analysis of fish health hazards should always be performed in three parallel areas:

- culture monitoring, which includes controlling water, fish behavior, growth, feed consumption, registering declines, applying defensive means (e.g., disinfectants);
- veterinary monitoring, official monitoring as part of supervisory programs as well as that performed by the veterinarian responsible for the facility;
- monitoring producers of commercial fish, including: systematic control studies of fish prior to sale and at the end of the production cycle, especially in doubtful situations or in those linked with actual or anticipated stress.

The husbandry of broodstocks is of great epidemiological significance. Spawners of sensitive species (both sexes) are examined routinely only as possible carriers of the VHS, IHN, and IPN viruses. Their condition should be monitored and also

evaluated with acute phase proteins, which are biomarkers of the early reaction of the organism to the penetration of pathogens. With regard to viral diseases, this is a very important turning point that permits the early identification of carriers of infectious agents in the ovarian fluid and in the milt, as transovarial transmission of IHNV and IPNV is possible (Wolf et al. 1963, Ahne 1983, Ahne and Negele 1985, Bootland et al. 1991).

Fish can have subclinical infections, which is why they are monitored during periods of potentially increased viral activity in spring and fall when temperatures are low (10-15°C), e.g., to detect infections of VHSV, IHNV, and IPNV. Studies of KHV are conducted in summer during periods of high temperatures (above 24°C), because the virus is active at higher temperatures. Breeding methods conducted on constant cell lines are the basis for isolating pathogenic viruses in fish, which is why the results are dependent on their activity in given periods. All of the factors that contribute to the immunological status of fish can impact interactions between the virus and the organism. The activity of humoral defense mechanisms is linked with the intense production of interferons. Sometimes fish succumb to viral diseases when they are in good condition, are feeding intensely, and are exhibiting good body growth. Even in these case the outbreaks of diseases can be sudden and acute with losses of 80-90% of stocks. It is also possible that latent infections can only be confirmed in the laboratory. The transmission of viruses by asymptomatic carriers (such as older fish, including wild specimens) and vectors species of fish that are not always subject to mandatory control studies pose significant threats to fish culture (Commission Regulation (EC) No 1251/2008, Johansen et al. 2011, Bernad et al. 2016a). Some pathogenic factors can negatively affect culture throughout the production cycle, e.g., infection with the IPNV, which can also predispose fish to higher mortality from bacterial diseases, i.e., yersiniosis in rainbow trout (E. Terech-Majewska, unpublished data). This could result from the impact that viruses have on the immune system, because viruses lower the activity of innate defense mechanisms, suppress metabolic and phagocyte killing activities, proliferative response of stimulated lymphocytes, serum lysozyme activity, and the immunoglobulin level (Siwicki et al. 1998c, Terech-Majewska et al. 2010c, 2016d). IPNV survives in the blood and kidney leukocytes, thus it can maintain a state of latent immunosuppression, then it becomes active when conditions are favorable (Maeda 2004, Matras et al. 2006). The IPNV is also released from decomposing dead fish and is excreted by diseased fish or asymptomatic carriers in excretions and secretions in quantities that are sufficient to be contagious (10 to 10^4 pfu ml^{-1}). In marine and fresh waters it can survive for 20 days at a temperature of 15°C (analogously, for 15 days at a temperature of 20°C) which facilitates IPNV persistence and its continued threat in coastal marine waters and its cycling through various culture systems. Among the three infectious agents monitored in salmonids, the IPN virus is designated as the most resistant to biocides and is also the most difficult to eliminate (Dixon et al. 2012). The most virulent strains can cause medium-sized rainbow trout kills of up to 40% of infected fish (Matras 2006, Terech-Majewska et al. 2011).

Prevention system that functions like this against the hazards posed by viruses does not include other infectious agents, such as bacteria, which are currently a much more significant problem, especially in terms of the quality of consumable food products that are obtained (Pękala 2010, Terech-Majewska et al. 2011, Pękala et al. 2015b). Bacterial and parasitic pathogens of fish are controlled through diagnostic tests that are conducted as part of what is called owner oversight. These include clinical diagnostics and viral, bacterial, mycological, and parasitological tests. Diagnostic procedures in the laboratories of the Veterinary Inspectorates and Departments of Veterinary Hygiene are supervised by PIWet (Kozińska et al. 2002, Kozińska et al. 2013). Aquaculturists can have basic tests performed at a laboratory of their choosing that is registered within the structure of the Departments of Veterinary Hygiene or other laboratories, including those that perform scientific research on fish disease diagnostics. The most frequently diagnosed problems caused by bacteria in

Poland include infections caused by *Aeromonas* spp. (*A. hydrophila*, *A. sobria*, *A. salmonicida* subsp. *salmonicida*, or atypical *A. salmonicida*), *Pseudomonas* spp. (*P. fluorescens*), *Yersinia ruckeri*, and *Flavobacterium* spp. (*F. psychrophilum*, *F. columnare*, *F. branchiophilum*). Losses are often the result of delayed diagnosis, the lack of systematic testing, and shifting susceptibilities to antibiotics that make treatment difficult (Kozińska et al. 2002, Terech-Majewska et al. 2008a, 2012b, Pękala 2010, Austin 2011, Bernad 2013, Pękala et al. 2015b, Bernad et al. 2016a, 2016b). Seasonal health problems are observed; for example, in spring the main problem is ectoparasites and stress-related diseases, e.g., columnaris (*F. columnarum*), *Aeromonas* spp. and *Pseudomonas* spp. infections (Kozińska and Pękala 2007, Bowden et al. 2007, Bernad 2013, Terech-Majewska and Siwicki 2013, Kaczorek et al. 2014, Bernad et al. 2016a). Seasonality could also stem from the period of increased stress in the spring-summer period, but also in fall, when fish are moved and subjected to other manipulations, e.g., prophylactic baths, body measurements, introducing new fish species to culture facilities. During these periods, there are changes in the natural environment (increased precipitation or the lack thereof) and in agriculture (field work, animal grazing). These can increase discharges of municipal sewage and pesticides into waters along with contaminated runoff from nearby fields. Natural immunity also exhibits seasonality; for example, trout immunity is lower in the winter and summer (Bowden et al. 2007). Quantitative-qualitative microbiological and biological water studies remain undervalued indicators in evaluating risk. Monitoring studies of water and fish from the Drwęca River and facilities located on this river demonstrated that the same microflora in differing quantities was detected in the water and on the skin of fish, which indicates that these types of studies are necessary (Lewandowska et al. 2004, Gołaś et al. 2009). These studies also indicated that the occurrence of the microflora differed depending on the origin (study site) and type (water or fish organs) of sample. *A. salmonicida* spp. *salmonicida*, *A. sobria*, *P. fluorescens*, and *P. putida* occurred in the different water and fish samples. Other microbiological studies that also examined healthy fish from selected Polish trout culture facilities operating at varying production intensities confirmed the dominance of the occurrence of these microorganisms in samples of internal organs taken from fish with high potential immunity (Terech-Majewska and Siwicki 2013, Terech-Majewska et al. 2012c, 2016d) (Figs. 2 and 3). Data from the literature indicate that the microorganisms belonging to those species are the natural microflora of aquatic basins as well as of healthy fish, which poses a permanent threat. The quantity of these microorganisms is variable and plays a significant quality role by co-creating a symbiotic setup that is important for homeostasis as well as the functioning of the immune system in fish. The pathogenicity of many factors (including viral ones, e.g., IPNV) can be the effect of relations among microorganisms and the biological properties of the environment (Maeda 2004).

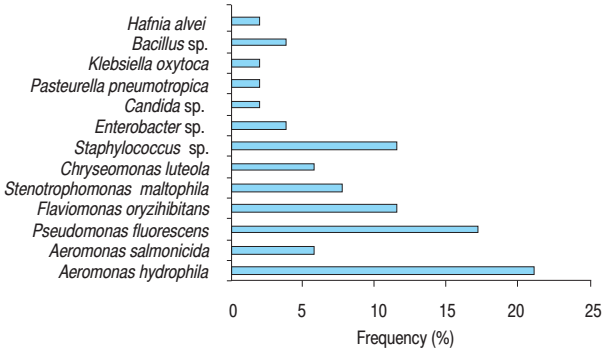

Figure 2. Frequency of occurrence of potentially pathogenic bacteria in rainbow trout reared in open systems.

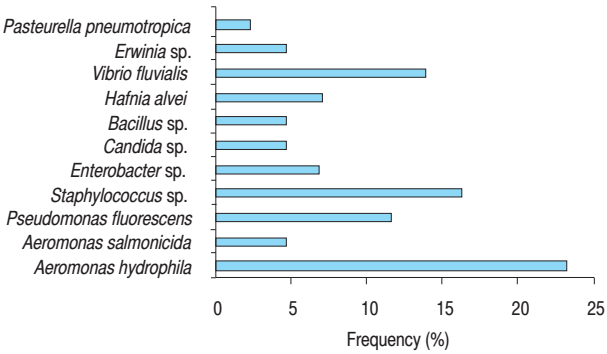

Figure 3. Frequency of occurrence of potentially pathogenic bacteria in rainbow trout reared in recirculating aquaculture systems (RAS).

Threats to fish health have to be considered according to species, even if there are many diseases that can be transmitted among several fish species, e.g., yersiniosis (*Y. ruckeri*) is a rainbow trout disease, but sturgeon and European eel are also susceptible to it. About 13 fish species have been found to be susceptible to infection by *Y. ruckeri*, as are some mammals (muskrat, *Ondatra zibethicus*) and birds (European herring gull, *Larus argentatus*). Humans (*Homo sapiens*) are also susceptible to this infection (Pękala 2010, Tinsley 2010, Sudheesh et al. 2012). Recently, new microorganisms have emerged that have only recently been isolated from clinical cases such as *Shewanella putrefaciens*, *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia*, and *Citrobacter freundii* (Kozińska and Pękala 2004, Bernad 2013, Kozińska et al. 2013, Pękala et al. 2015a, Bernad et al. 2016a).

New strategies for the prevention and treatment of fish diseases recommend including quantitative-qualitative monitoring of the microbiological contamination of waters to identify the threat posed to fish. This appears to be a significant element of risk evaluation, particularly in closed systems, in which the analysis of the microbiomes is important to specific species as well as to the technology of fish culture (Dulski et al. 2016). During critical periods, these studies could provide the foundation for targeted immunoprophylaxis.

It is difficult to isolate culture facilities from the natural environment, which is inhabited by potential disease carriers and the pathogens of vector species. Even in closed systems and those that are totally isolated from the natural environment, pathogens that can be introduced through feeds or by humans are detected (E. Terech-Majewska, unpublished data). It is difficult to isolate facilities from water-borne hazards such as pesticides, heavy metals, detergents, and biocides. Such contaminants can contain elements with known immunotoxic, toxic, and carcinogenic properties (Rymuszka et al. 1998, Rymuszka and Siwicki 2004, Terech-Majewska et al. 2008b, 2015c, Parol et al. 2015, Tkachenko et al. 2015b).

Environmental hazards to fish health and culture errors

In addition to infectious agents, non-infectious ones, which are often much more difficult to eliminate and can also be hazardous to fish health, occur in controlled culture (Table 2). All types of fish culture are susceptible to this, including those in recirculating systems, in which the effects can be intensified because of the limited space within which they circulate. The environmental parameters of water, including, among others, temperature, pH, oxygen content, hardness, and nitrogen and phosphorus content, have to be monitored continually in all types of fish culture. Performing obligatory tests twice annually, which is required for legal-water permits, is insufficient since the aquatic environment is highly variable (Regulation of the Ministry of Environment of July 24, 2006). Toxicological and biological monitoring of water is necessary for the prevention and treatment of fish diseases since it can identify processes occurring in this environment (Nan et al. 2009, Sidoruk 2012, Sidoruk et al. 2013, Bogusławska-Wąs 2015). Health problems in fish linked with non-infectious agents include excessive fat deposition, vitamin and mineral deficiencies, and poisoning (Siwicki et al. 1994a, Antychowicz 2007, Noga 2010). Excessive fat deposition is fairly common in fish (especially in spawners) that are fed large quantities of fats with a simultaneous deficit of vitamins and microelements. Increased deposits of fatty tissues around internal organs, especially the heart, leads to circulatory disorders and liver degeneration that can lead to metabolic and endocrine disruptions. Vitamin deficiency is a side effect of inappropriate vitamin levels in the feed. Deficiencies can also be caused by gastrointestinal tract inflammation resulting from chronic viral and bacterial infections and parasitic infestations that cause physical damage to mucus membranes that lead to chronic inflammation. The elimination of desirable saprophytic bacterial flora in the digestive tract during or following antibiotic treatment is also a cause of such deficiencies. This has a direct impact on proper metabolism and organ function. Freshwater fish are prone to

Table 2
Critical factors in the controlled rearing of fish, their impact on the fish, and possibilities for reducing their impact

Critical factors	Stress	Anti-stress remedies	Weakened immunity	Immunomodulators	Vaccinations
Changing water parameters temp., pH, O_2, others	+++	+++	+/-	+/-	+/-
Water flow	+++	+++	+/-	+/-	+/-
Water turbidity/clarity	+++	+++	+/-	+/-	+/-
Water blooms	+++	+++	+/-	+/- a minimum of two weeks prior to blooms	+/- a minimum of two weeks prior to blooms
Changing feed/feed quality and composition	+++	+++	+/- feed contamination	+/- limited effect	+/- limited effect
Moving fish/transport	+++	+++ if administered prior to and during transport	+/-	+/- administered two weeks prior	+/- administered two weeks prior to change, but can also be administered during the moving of fish and during transport if performed in accordance with the requirements of that stage
Microbiological quality of the water stemming from natural processes in the aquatic environment	+/-	+/-	+++ high risk if the quantity of microorganismsis excessive *E. coli* (nL^{-1}) < 2500 to 5000 is safe	+/- depends on causative agent and immunological condition	+/- depends on causative agent and immunological condition
Water mineral quality	+/-	+/-	+++	+/- immunomodulators with mineral supplements as feed supplements	+/-
Sanitary and chemical runoff	+++	+/-	+++	+/- depends on the length of exposure to and concentrations of compounds in the water	+/-
Wild fish and animals	+/-	+/-	+++ if they are the source or vectors of infectious agents	+/-	+/-

overhydration since they inhabit a hypotonic environment and lose ions through the gills. Freshwater species drink only small amounts of water and need more minerals in their diets. Deficiencies in Na, Cl, K, Ca, Mg, Zn, Mn, Cu, J, Se, and Cr are particularly important for immunity. These minerals are taken up from the water and feed, while water and feed quality and the physiological status of the fish impact their utilization (Brucka-Jastrzębska et al. 2013, Pajdak et al. 2015, Terech-Majewska et al. 2016b). Water conductivity, which indicates indirectly the water ion content, is only monitored in closed systems. In other fish culture technologies this type of test is performed very rarely even though knowing what the water mineral qualities of a given facility are could help to lessen the impact these agents have on the bodily functions of fish. Different tissues differ in the content of elements, and contents of them in organs is a species characteristic (Brucka-Jastrzębska et al. 2009, 2010). For example, iron contents in particular organs in trout and carp are as follows: decreasing amounts of iron in trout are noted in the kidneys>liver>gills>blood->muscles>skin, while in carp, analogously, in the gills>kidneys>blood>liver>muscles>skin. The health status of fish also impacts the content of micro- and macroelements in tissues, and during the course of diseases requirements for them in the tissues or their distribution are disrupted (Pouramahad and O'Brein 2000).

Monitoring the levels of micro- and macroelements in fish tissues is also of diagnostic significance as it permits observing pathological changes early on. Changes in the levels of elements appear quickly and are preceded by other symptoms such as changes in fish behavior or visible damage from disease (Brucka-Jastrzębska et al. 2009). When there is a lack of elements in the water, the fish must be given supplements in the feed, because only then are the effects of deficiencies not observed (Wood et al. 2012, Barszcz et al. 2014a, 2014b). Microelements, for example zinc, are especially important since they are directly associated with the proper functioning of the immune system. Zinc dietary supplementation for trout should be correlated with the level of this element in the water, e.g., at 11 μg Zn L^{-1}, the feed supplement should be from 15 to 30 mg kg^{-1} feed (Terech-Majewska et al. 2014d).

Antioxidant enzyme activity and non-enzymatic antioxidant, or mineral, content is codependent in fish. It has been demonstrated that the place and conditions of fish culture are correlated with antioxidant indicators and infection susceptibility. Trout diagnosed with viral or bacterial infections present lower antioxidant enzyme activity in the kidneys, liver, and blood (Brucka-Jastrzębska et al. 2013). Enzymatic and non-enzymatic antioxidants permit maintaining balance between their activity and the quantity of free radicals released in fish cells under the influence of factors in both external and internal environment.

Aspects of toxicology assume specific significance when fish are poisoned, which is relatively infrequently confirmed. Chemical agents are much more damaging in sub-threshold quantities or when they accumulate in bodies and impact them over long periods of time. The state of the aquatic environment and its stability determines the occurrence of infectious and non-infectious diseases. Non-specific symptoms including fin and gill necrosis, skin ulcers, or ecchymosis in fish can be associated with the impact of municipal and industrial wastewater and crop protection products, which must be excluded each time a differential diagnosis is made and absolutely must be considered when evaluating risks posed to health. Organochlorine insecticides and organophosphorus herbicides, aromatic hydrocarbons, pentachlorophenol, heavy metals, and chemotherapeutics are particularly toxic for fish. Biodegradation processes that are continual in the aquatic environment determine the bioavailability to fish of these compounds, which have adversely affect the endocrine or immune systems or directly disrupt the functioning of various organs (Anderson and Siwicki 1996, Rymuszka et al. 1998, Siwicki et al. 1998e, 2010c, Studnicka et al. 2000, Terech-Majewska et al. 2003, 2008b, Kolman et al. 2003, Rymuszka and Siwicki 2004, Rico et al. 2012, Kaczorek et al. 2015).

Organophosphates, pyrethroids (e.g., cypermethrin), and derivatives of triazines (e.g., atrazine) are the most widely used pesticides in agriculture.

Cypermethrin is much more toxic to fish (LC50 0.4-2.8 μg L^{-1}) and aquatic organisms (LC50 0.01-5 μg L^{-1}) than it is to mammals (LC50 for rats 400-800 mg kg^{-1}). Atrazine, a commonly used herbicide, is detected in samples of surface water (50%) and even in groundwater (10%) in Europe. In vitro studies have shown that at concentrations of 10, 50, and 100 μg L^{-1} innate immunity is impaired (Rymuszka et al. 1998). Disruptions in the immune system such as reduced phagocytosis, reductions in the number of antibody-producing cells, decreased cell proliferative capacity, and decreased lysozyme activity can be caused by heavy metals (Al, As, Cd, Chr, Cu, Pb, Hg, Ni, Zn), aromatic hydrocarbons (benzidine, polychlorinated biphenyls, phenols), pesticides (dichlorvos, DDT, trichlorfon), mycotoxins (aflatoxin, fumonisin), and antibiotics (florfenicol, oxolinic acid, oxytetracycline) (Gleichmann et al. 1989, Siwicki et al. 1996c, 1998e, 1999b, 2010d, Szarek 1999a, 1999b, Lunden and Bylund 2000, Sierosławska et al. 2000, 2004, 2005, 2007, Studnicka et al. 2000, Sierosławska and Siwicki 2003, Terech-Majewska and Siwicki 2006, Wojtacka 2007, Rymuszka and Adaszek 2013, Sierosławska and Rymuszka 2013). Immune system dysfunction is sometimes a consequence of pathological lesions that are observed in the organs and in lymphoid tissues (Szarek et al. 2000a, 2000b, Wojtacka et al. 2011, 2015).

New fish species in aquaculture and health hazards

The common whitefish is a salmonid (*Salmonidae*) that is endemic to Polish waters. In the 1970s, it occurred in more than 270 lakes, and catches of it exceeded 100 tons (Szczerbowski 2000), but this species is currently threatened with extinction in Poland (IUCN 2001, Kowalska and Zakęś 2010). The occurrence of this species in natural habitats is dependent on proper fisheries management and the production of stocking and rearing material in controlled culture. The taxonomic status of the whitefish population inhabiting Polish lakes and Baltic coastal waters is not fully known. It is possible that the majority of the endemic whitefish population is, in fact, hybrids that are the consequence of hybridization and the transfers of stocking material (Martyniak et al. 2004, Fopp-Bayat and Wiśniewska 2010, Polewacz et al. 2015). Whitefish is cultured using material obtained from artificial spawning (wild and cultured spawners) as well as hatchlings and summer and fall fry (Szczepkowski et al. 2008, Hliwa et al. 2010, Mickiewicz et al. 2011, Szczepkowski 2011). One condition for good production was devising a feeding program based on determining optimized feed, daily feed ration, proximate of the feed, and how the feed is delivered (Szczepkowska et al. 2007, Wunderlich et al. 2010, Szczepkowski 2011). The whitefish is very sensitive to adaptive and manipulation stress since it sheds scales easily, which is why it is the most advantageous to leave it in the same ponds from stocking until they reach commercial size. When changes in temperature are gradual, it can tolerate extremely high temperatures (for a cryophilic species) as high as 25-27°C. It is very sensitive to rapid temperature changes e.g., from 18 to 12°C. Health problems are usually seen in fall fry and older fish weighing from 80 to 90 g (Terech-Majewska et al. 2011). The prophylaxis used in controlled whitefish culture is similar to that used for rainbow trout or grayling, i.e., bathing spawn, hatchlings, and fry in biocides at lower concentrations (Oxyper, chloramine-T, formalin, Steridial), (Terech-Majewska et al. 2011, Grudniewska et al. 2014). Evaluations of the health of fish used in restocking are done according to the general requirements for protecting the health of animals in aquaculture that aim primarily at limiting the spread of VHSV and IHNV, because the common whitefish is one of the natural vectors of these diseases (Skall et al. 2004, Council Directive 2006). Additionally, this species if potentially susceptible to infections caused by *Aeromonas* spp., *Pseudomonas* spp., and *S. putrefaciens* (Terech-Majewska et al. 2011). Bacterial diseases are diagnosed most frequently in the fall fry stage during periods of significant, abrupt changes in temperature. During

P. fluorescens infection, skin ulcers of varying severity appear, while *S. putrefaciens* generalized infections cause changes to the internal organs (pale kidneys, hepatomegaly, liver marbling, general ecchymosis). External parasites occur primarily in summer fry and include *Trichodina* sp., *Chilodonella* sp., *Apiosoma* sp., *Trichophyra* sp., *Ichthyophtirius multiphiliis*, *Dactylogyrus* sp., and even *Proteocephalus* sp, while in fall fry infections of *Argulus* sp. have been confirmed (Terech-Majewska et al. 2011).

Pikeperch is a very attractive fish intended for both restocking and consumption, and the culture of it is being moved to farming in RAS (Zakęś 2009). Depending on the conditions under which this species is being cultured, it is susceptible to many parasites, bacteria, and viruses. The bacteria that are isolates most frequently include *A. sobria*, *P. fluorescens*, *P. putida*, and *Chryseobacterium luteola* (Zakęś 2009). As a thermophilic fish, it requires a controlled rearing temperature of 22°C. This is also a temperature that is preferred for a wide range of conditionally pathogenic microorganisms. Relatively little is known about pikeperch susceptibility to viral infections; however susceptibility to infection has been noted following injection with several types of viruses that are known pathogens of other fish species with similar environmental requirements, e.g., epizootic hematopoietic necrosis virus (EHNV) and European sheatfish virus (ESV) (Jensen et al. 2011). External parasites that generally occur on summer fry include the following: *Trichodina* sp.; *Chilodonella* sp.; *Ichthyobodo* sp.; *Ichthyophthirius multiphiliis*; *Gyrodactylus* spp. However, the most problematic health challenges are metabolic and developmental disorders, i.e., spine anomalies, swimbladder inflation (in the second week of rearing), edema, jaw anomalies (throughout rearing), eyeball and lateral line anomalies. Critical points during controlled culture include: the transition to exogenous feeding (several days after hatching), swimbladder inflation (between days 4-11 after hatching), and the period of increased cannibalism (10-20 mm in size). Only after the fish have attained a body weight (b.w.) of 15 g can fattening begin (Zakęś 2009). Various feeding procedures using feeds that are fortified with natural and/or synthetic supplements that are administered to optimize growth and health and to increase the possibilities of intensifying production are used in closed systems. Growing knowledge on the immune mechanisms of this fish species also permits using immunomodulation in its controlled rearing (Siwicki et al. 2003b).

European eel is a valuable fish species in European fisheries management. The development of the industrial-scale production of restocking material and commercial fish has become possible thanks to the development of controlled culture and fattening technologies especially in RAS (Robak and Przystawik 2007, Robak et al. 2007). Although European eel is known to be very resistant to a range of factors, it is still susceptible to infection from a few, specific types of viruses, i.e., eel virus European (EVE), anguillid herpesvirus 1 (AngHV-1), and the rhabdovirus eel virus European X (EVEX) (syn. eel virus American – EVA) (Davidse et al. 1999, van Ginneken et al. 2004, Haenen et al. 2012). Infections caused by a few viruses simultaneously can be particularly hazardous. The risk of the spread of viruses occurs during migrations, at culture facilities, and during the sale of live fish. Another significant cause of losses in eel populations is bacterial infection, i.e., *P. anguilliseptica*, *Vibrio vulnificus*, *Plesiomonas shigelloides*, *A. hydrophila*. Infections with the following are noted less frequently: *A. jandanei*; *P. fluorescens*; *S. putrefaciens*; *A. sobria*; *A. caviae*; *A. salmonicida*; *Flavobacterium* spp. These microorganisms occur in both the natural environment and culture facilities. Bacterial flora that accompany fish in fresh waters include *Pseudomonas* spp., *Moraxella* spp., and *Acinetobacter* spp. *Escherichia* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., and *Streptococcus* spp. have also all been isolated from healthy fish (Esteve and Garay 1991, Pedersen et al. 1999, Sudheesh et al. 2012).

Fifty-five species of parasite have been identified to date on wild, healthy eel, including four species of external and 16 internal trematodes, six species of cestodes, and 21 species of nematodes (Dzika et al.

1995, Własow et al. 1996, Kennedy 2007). The number of parasites isolated from eel inhabiting natural waters depends on the location and period in which samples are collected. The most frequently identified parasites on eel in culture facilities are those with a simple developmental cycle, i.e., protozoa and monogenean (*Platyhelminthes*). Parasites that form cysts or deposit eggs and can survive in culture systems even during technological production breaks are the focus of monitoring studies. Those most frequently confirmed are: the protozoan *I. multiphillis*, the ciliate protists *Trichodina* spp., and monogenean platyhelminths of the gills (*Dactylogyrus* spp., *Pseudodactylogyrus* spp.) (Buchmann et al. 1987, Madsen et al. 2000, Terech-Majewska et al. 2016c). Critical points during the production cycle include the adaptation period and the rearing of glass eel from the fry stage. Studies by Siwicki and Robak (2011d) indicate that the indices of innate immunity in cultured fish are low in comparison to those of fish caught in natural basins. Higher levels of total protein in cultured fish indicate the optimum balanced level; however, this disproportion could predispose them to infection after they are released as stocking material. Generally, eel are considered to be relatively easy to rear.

Silurids command a lot of attention as they comprise species that can be cultured at high levels of intensity while also being tolerant to varying environmental conditions. Diseases do occur in controlled rearing to which European and African catfish are especially susceptible. High water temperatures that support viral and bacterial agents facilitate fish infection. The most hazardous viruses for these fish belong to the epizootic hematopoietic necrosis virus group (EHNVG) that includes the epizootic hematopoietic necrosis virus (EHNV) isolated from perch and rainbow trout in Australia (a second species of EHNV was isolated from wels catfish) and iridovirus isolated from black bullhead catfish. This disease occurs most frequently in the spring-summer period and also in facilities with heated waters. This pathogen is isolated from the liver, spleen, and gastrointestinal tract of fish, while the organs for these trophic agents are the cephalic kidney cells in which hematopoietic necrosis develops. This virus causes substantial damage in the body, i.e., focal necrosis of the liver, cephalic kidney, and spleen. In the decided majority of cases, this infection leads to distention in the abdominal wall, and fish stop feeding, become lethargic, and slowly come to rest on their sides. Experimental infections indicate that the virus can penetrate through the alimentary canal after *per os* infection through immersion or injection. The rate at which the disease develops (from 72 to 96 h) depends on the condition of the fish and the temperature of the water. High fish mortality (80-100%) develops over the course of five to seven days (Kazuń and Kazuń 2010). Silurids succumb to disease in intense culture conditions when these factors generate immunosuppression, can predispose the fish to development other diseases. In in vitro studies of leucocytes isolated from the cephalic kidney of catfish that were performed at temperatures of 20 and 30°C, Siwicki et al. (1999a) demonstrated the suppressive impact of the virus on phagocyte metabolic activity (more distinct at a temperature of 30°C). Additionally, the proliferative activity of lymphocytes stimulated with concanavalin A (ConA) and lipopolysaccharide (LPS) was reduced. Siwicki et al. (2001e) also demonstrated that the virus causes a decreased metabolic activity of macrophages isolated from cephalic kidneys of carp, trout, and catfish and incubated under the same conditions; the least significant effect on the parameters tested was observed in trout cells.

Enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri*, is another disease that this group of fish is at risk of contracting. It occurs most frequently in a temperature range of 18-28°C, and its development is facilitated by inappropriate living conditions including excessive stocking density, manipulation stress, or poor water quality. In addition to septicemia, chronic encephalitis that is granulomatosic in character (so-called hole-in-the-head disease). This bacterium remains in the kidneys for approximately four months, which facilitates prolonging the presence of the diseases in culture facilities.

Other diseases that are less typical of silurids include columnaris (*F. columnare*), edwardsiellosis (*E. tarda*), and many others. When reared in intense culture, the skin and alimentary tract of these fish are inhabited primarily by microorganisms of the genera *A. hydrophila*, *P. fluorescens*, *Stahylococcus* spp. and other *Enterobacteriaceae*, which can also pose risks to fish health (Harnisz et al. 2004).

Chemical and antibiotic therapies in fish culture

Biocides and antibiotics therapies are used widely and are the most frequently therapies used in fisheries especially against pathogens that permanently present in the vicinity of fish (Siwicki et al. 1994a, Terech-Majewska et al. 2004b, 2005, 2010a, Grudniewska et al. 2006, 2014, Antychowicz 2007, Własow and Guziur 2008, Noga 2010, Harnisz et al. 2011, Grudniewska and Terech-Majewska 2015). Today, the use of these preparations is controversial when they are administered with the feed (without cover) or in baths that are released directly into the environment that can cause disadvantageous phenomena (Jakobsen 1988, Hektoen 1993, 1995, Klaver and Matthews 1994, Kerry et al.1995, Terech-Majewska et al. 2012a, 2014a, Gothwal and Shashidhar 2015). Drug traces in the edible tissues of fish is a problem associated with responsibility for consumer health and the disadvantageous effect on the animal that has been treated. These effects include damage to organs in which high concentrations accumulate such as the kidneys or liver, digestive tract disorders, and superinfection with other pathogens. They can also have an impact on the functioning of the immune system cells. Studies conducted by many researchers on the chemotherapeutics effects on the immune system, have indicated that they negatively impact cellular and humoral defense mechanisms in fish (Ingram 1980, Gnarpe and Belsheim 1981, Grondel 1985, 1987a, Gleichmann et al. 1989, Dunier and Siwicki 1994, Radomska 1994, Siwicki et al. 1998d, Lunden et al. 2002, Sierosławska et al. 2004, Terech-Majewska and Siwicki 2006, Kum and Sekkin 2011). Antibiotics do not eliminate microorganisms from the body; they either kill them (bactericidal activity) or suppress their growth (bacteriostatic activity). Without the appropriate elements of the immune response, the effective elimination of infection is impossible. The drugs of choice in aquaculture are tetracycline, quinolones, aminoglycosides, and sulfonamides. It is especially important to be careful when using antibiotics that persist in the aquatic environment the longest, migrate through the trophic chain, and potentially generate antibiotic resistance in the sediments (Gothwal and Shashidhar 2015, Pękala et al. 2015b). An additional challenge in closed systems is protecting the biofilter microenvironment, upon which the equilibrium of water chemical parameters depends (Bogusławska-Wąs 2015). The most widely used antibiotics in aquaculture are tetracycline and quinolones.

Tetracycline is a broad-spectrum antibiotic that is highly effective, which is why it was one of the first antibiotics used in the treatment of cultured fish. Oxytetracycline (OTC) is the most important drug from this group used to treat fish as it is effective against most microorganisms that are pathogenic in fish, namely *Flexibacter* sp., *Vibrio* sp., *Aeromonas* sp., *Yersinia* sp., and *Edwardsiella* sp. Tetracycline is considered to be an immunosupressant (Ingram 1980, Lunden and Bylund 2000, Terech-Majewska et al. 2006, Wojtacka 2007). Impairs phagocytosis, mechanisms of intracellular killing, and T-lymphocyte cytotoxicity in both humans and animals (Radomska 1994). The impact of tetracycline on phagocyte function could affect both the absorption stage and intracellular killing. Tetracycline effects are dependent on drug concentration, the length of cellular exposure, and the amount of calcium ions in the environment. OTC suppresses the formation of F-actin, a cytoskeletal protein that determines cell motility through calcium chelation (Ca^{2+}) that interferes with mechanisms responsible for the formation of these elements (Gleichmann et al. 1989). Binding intracellular calcium ions inhibits phagosome maturation thus preventing proper phagocytosis (Myers et

al. 1995). In fish, as in mammals, stimulating phagocyte cell membranes activates NADPH oxidase and the production of reactive oxygen species that have germicidal properties (i.e., RBA – Respiratory Burst Activity reaction). OTC suppression of free radical production is observed *in vitro* in different fish species, while statistically significant effects are observed at doses ranging from 0.1 to 100 μg ml^{-1} (Lunden et al. 2002). During treatment, organs rich in phagocytes, e.g., the cephalic kidney, tetracycline reaches high concentrations that far exceed those in the serum (Grondel et al. 1987a, 1987b, Łapińska et al. 2005). Tetracycline can also disrupt resistance processes in which lymphocytes are engaged. Doses of the antibiotic of 6 μg ml^{-1} limit the incorporation of 3H-thymidine into carp lymphocyte DNA stimulated with PHA (phytohemagglutinin), which is evidence of the inhibitory effects of the drug on the ability of the T-cell proliferative response to the antigen (Grondel et al. 1985). In *in vitro* and *in vivo* studies of rainbow trout fry, Lunden et al. (2000) demonstrate the suppression of activity in the proliferation of cells isolated from the cephalic kidney by 40% of B-lymphocytes and more than 60% of T-lymphocytes. This effect was achieved at a dose of 75 mg kg^{-1} b.w. *per os* administered for 10 d. Administering OTC with the feed is recommended, and, in practice, this is usually how it is done at doses of up to 100 mg kg^{-1} b.w. for 10 d (Siwicki et al. 1994a, Antychowicz 2007, Noga 2010). OCT is thought to impact the lymphocyte proliferation rate in two ways. Firstly, it is a calcium ion chelator that decreases the quantities of this element in the vicinity of cells, thus reducing the inflow of Ca^{2+} to lymphocytes after the stimulation of cells by mitogens, which inhibits the synthesis of DNA and RNA (Grondel et al. 1985, Myers et al. 1995). The second interaction that could occur even at low concentrations of OTC is based on impaired mitochondrial protein synthesis, which disrupts mitochondrial biogenesis (Kwiatkowska and Sobota 1999). Tetracycline effects bacterial cells by binding with the ribosomal 30S subunit and inhibiting microorganism protein synthesis. At concentrations >20 g ml^{-1} it can block mitochondrial protein synthesis in eukaryotic cells, and at concentrations >50 g ml^{-1} it can block cytoplasmic protein synthesis (Lunden and Bylund 2000). Disruptions in mitochondrial protein synthesis result in lower levels of the enzymes that participate in cell division, lead to a weakened ability or even inability of cells to proliferate, and reduce cell metabolic potential. One effect after administering OTC can be the suppression of antibody production (Grondel et al. 1987a, 1987b).

Terech-Majewska and Siwicki (2006) determined the impact of oxytetracycline on the metabolic activities (RBA) and phagocytosis activity (PKA – potential killing activity) of polymorphonuclear (PMN) and mononuclear (MN) leukocytes and the proliferative response to T and B cells (MTT – mitogenic transformation test) in wels catfish and carp after a single intraperitoneal injection of 10 mg kg^{-1} b.w. Lowered RBA and PKA values were noted between days 4 and 14 after OTC was administered, and on about day 21, the parameters returned to the initial values. Statistically significant decreases in MTT ((lymphocytes stimulated with the mitogens concanavalin (Con A) and lipopolysaccharide (LPS)) was noted between days 4 and 12 after OTC was administered. The period of immunosuppression in wels catfish that were administered OTC was similar, and the RBA, PKA, and MTT parameters differed only slightly from those of carp. Continued immunosuppression for a few days following a single 10 mg kg^{-1} b.w. dose of OTC could foster repeat infections and decease the adaptive potential of fish.

OTC does not biodegrade readily in aquatic environments, especially not in basins with large quantities of sediments (Jacobsen and Berlind 1988, Samuelsen et al. 1992, Kerry et al. 1995). Complexes with Ca and Mg ions form in bottom sediments which not only hinders biodegradation but also facilitates the long-term presence of the antibiotic in the environment and increased drug resistance (Austin 1985, Hektoen et al. 1993, 1995). Tetracycline is confirmed in the sediments during the period that fish are administered the antibiotic (up to 100% of sediment samples tested), and 18 months following its use (in 10-50% sediment samples tested) (Samuelsen et al. 1992, Łapińska et al. 2005). Under experimental conditions, tetracycline was confirmed

to inhibit water physicochemical transformations that could lead to fish poisoning, especially with nitrogen compounds (Klaver and Matthews 1994). Residues of tetracycline were also detected in the tissues of wild fish and in zooplankton near a facility where fish had been treated with this drug (Ervik et al. 1994).

First-, second-, and third-generation quinolone are highly effective against a range of fish pathogens, especially Gram (-) bacteria. Their effectiveness against microorganisms and fish depends on the dose and the length of time the drug is administered. They are most frequently administered *per os* in doses of 10 to 20 mg kg^{-1} b.w. for 5 to 10 d or in baths at concentrations of 2 to 100 mg L^{-1} water in a wide range of temporal configurations (Antychowicz 2007, Noga 2010). These antibiotics are considered to have the least impact, relatively, on defense mechanisms. It has been confirmed that they can lower the metabolic activity of neutrophils through the activation of myeloperoxidase (Lunden et al. 2002). Sierosławska et al. (2005, 2007) did not observe lowered metabolic or killing activity following intraperitoneal administration of norfloxacin (NNOR). NNOR caused a reduction in T- and B-lymphocyte proliferation activity as well as lowered levels of antibody-producing cells in rainbow trout vaccinated against *Y. ruckeri* infection following the administration of NNOR. Quinolones, similarly to tetracycline, are Ca^{2+} ion chelators, and they can impact ion concentrations both outside and inside lymphocytes. This could affect proliferation activity stemming from disruptions in the availability of Ca^{2+} for immune cells. At high doses, as an agent that blocks bacterial enzymes responsible for processes of replication and transcription, they can hinder DNA synthesis in rapidly dividing eukaryotic cells (Sierosławska et al. 2004). Florfenicol (FF), which is often the drug of last resort, is frequently used to fight ulceration in salmonids. Like other antibiotics from this group, FF can act in various ways on immune cells. Its metabolites bind to melanin and accumulate in the cephalic kidney macrophages. Traces of metabolites are detected in fish tissues for a long time even after a single dose, e.g., in Atlantic salmon after administering 20 mg kg^{-1} b.w. residues are detectable for eight weeks. This might indicate that the elimination of the antibiotic from the body requires a long time. The presence of the antibiotic in the bodies of treated fish can also cause a reduction in the proliferative capacity of T- and B-lymphocytes.

Defense mechanisms and anti-infection immunity fish

The immune system (IS) is highly conservative across species, and despite certain anatomical and physiological differences stemming from the lower degree of phylogenic development in fish, it functions according to similar principles in all vertebrates and humans (Stosik and Deptuła 1990, Siwicki et al. 1999c, Van Muiswinkel and Nakao 2014). The functioning of the IS is dependent on the maturity of its structure. The hematopoietic organs in fish include the thymus, spleen, cephalic (pronephros) and trunk (mesonephros) kidneys, while the lymphohematopoietic functions mostly regard the cephalic kidney, which, in contrast to the trunk kidney, has no excretory function. Cells comprising the lymphoid group are located in both the epithelium and lamina propria mucosae, known as the gut-associated lymphoid tissue (GALT). Upon activation by a pathogen, the immune response, depending on its type, is divided into cellular and humoral. However, each immune response usually comprises both of these elements. Immune mechanisms can by divided into innate (non-specific – independent of the type of pathogen) ad adaptive (specific – dependent on the type of pathogen), which is formed under the influence of antigenic stimuli.

Innate immunity is associated with, among other things, the occurrence of natural anatomical and physiological barriers such as the continuous outer skin and the mucous membranes. Significantly, fish lack skin keratosis, and their epidermal cells proliferate rapidly so skin lesions heal very quickly (Stosik and Deptuła 1990). Another component of this system is the mucus that is excreted by the cells of the

epidermis, gills, and gastrointestinal tract. Elements of innate humoral response include the complement, properdin, lysozyme, and C-reactive protein agglutinin, precipitin and lysine (belonging to IgM) found in the serum and mucus, as well as interferons, ceruloplasmin, transferrin, and chitinase. The elements of the innate cellular response include MN cells – monocytes and/or macrophages – and PMN – neutrophils – the functions of which are evaluated with phagocytosis potential, cytotoxicity, and cytolysis. The functioning of adaptive immunity mechanisms is dependent on the activity of the T- and B-lymphocytes of the thymus and cephalic kidney and is conditioned by their products. For example, T-lymphocytes secrete lymphokines, e.g., MIF – macrophage migration inhibitor factor. Specific humoral immunity is conditioned by immunoglobulin produced by B-lymphocytes.

The skin and the mucous membranes of the gastrointestinal and respiratory (gills) tracts are the primary point of contact between fish and the external environment. Currently, attention is being focused on the immune system associated with the mucus membranes of the gastrointestinal tract (GI tract), the absorption surface of which is about fifteen times larger than that of the skin. This system is exposed to environmental antigens and pathogenic agents to a far greater degree than are the gills. Functioning in the mucus and submucus membranes are well-organized clusters of lymphatic nodules, known as mucosa-associated lymphoid tissue (MALT). This is the principle element of the immune system in the GI tract, which is known as GALT. The primary function of the mucus membrane lymphatic system is to create antibodies (IgM) that enter secretions as secretory IgM (S-IgM). Antibodies play active roles in various reactions, i.e., encapsulating and agglutinating pathogenic microorganisms, bacteriostasis, preventing microorganisms from adhering to the epithelium and penetrating into the mucosa, and neutralizing bacterial toxins. Each antigenically active element that reaches the GI tract can be used to induce systemic resistance. The result of activation is the prevalence of activated effector plasma cells that produce specific antibodies in various areas of the mucus membranes and associated tissues (Van Muiswinkel and Cooper 1992, Press and Evensen 1999, Van Muiswinkel and Nakao 2014).

The most effective defense barrier in the GI tract is the microenvironment and the components of the digestive mucus, i.e., the low pH of the digestive fluids, proteolytic enzymes, lysozyme, lactoferrin, defensins, mucus, and the physiological (saprophytic) bacterial flora. The cylindrical epithelium that contains absorbent cells, i.e., enterocytes, goblet cells, and intraepithelial leukocytes is an impenetrable barrier. A special role is played by the defensive cells at the base of intestinal glands, because they contain potent antimicrobial proteins like lysozyme, secretory phospholipase A2, and defensin (crypdins). These are secreted when there is direct contact between these cells and bacteria or with the components of bacterial walls. The normal microflora occurring in the GI tract plays an important role in mucus membrane immunity by creating its own microbiome, a microenvironment that is very sensitive to changes (Dulski et al. 2016). The saprophytic bacterial microflora prevents the growth of pathogenic bacteria and even increases resistance. The following mechanisms are especially important: competition for nutrients, competition for receptor sites and adherence to the intestinal epithelium, stimulation of natural antibodies, production of bacteriocin (colicin) by physiological microflora, which comprise the GI tract immune system as killing agents of pathogenic bacteria (Sahoo et al. 2014).

From the point of view of culture practice, the possibility of evaluating the impact of various pathogenic and environmental factors on the functioning of the immune system is important. Many indicators have been developed which can be used to analyze immune system function and also for evaluating prophylactic procedures applied (e.g., vaccinations, disinfection) (Mosmann 1983, Magnadotiir 2000). Most frequently the aim of examining the immune response is to evaluate the following: 1) the metabolic activity of the phagocytes isolated from the spleen/cephalic kidney/blood by evaluating the level of intracellular RBA after stimulation with phorbol myristate acetate (PMA) (Siwicki et al. 1996a); 2) the intracellular PKA isolated

in the spleen/cephalic kidney/blood against infectious agents (Siwicki and Anderson 1993); 3) the proliferation activity of T-lymphocytes isolated from the cephalic kidney and stimulated with the mitogen ConA (Siwicki et al. 1996a); 4) the proliferation activity of B-lymphocytes isolated from the cephalic kidney and stimulated with the mitogen LPS (Siwicki et al. 1996a); 5) The lysozyme activity in the serum using *Micrococcus lysodeikticus* bacteria with the turbidimetric method, as modified by Anderson and Siwicki (1993b); 6) the activity and myeloperoxidase (MPO) activity in the PMN cells (Siwicki et al. 1994a); 7) total protein (TP) with the biuret assay using the Diagnostic Kits Protein Total Reagents test (Sigma) (Siwicki and Anderson 1993).

Many other methods are used in addition to those already mentioned as immune system testing methodology is developing intensively. Methods have been introduced that permit determining the levels of B-lymphocytes and their ability to identify antigens and create antigen-presenting cells (APC) (Siwicki and Dunier 1993) and also to determine ceruloplasmin levels (Siwicki et al. 1993). With the aim of evaluating the effects of actions taken and also to evaluate the impact of immunomodulators, challenge tests (ChT) are used. In these tests the impact of selected agents on the survival rate of fish following experimental infection with bacterial strains that are recognized as pathogenic is studied (Siwicki et al. 2010e). The culture indicators, such as coefficients of condition and growth and the hepatosomatic, spleen somatic and viscerosomatic indexes (Kowalska et al. 2015, 2016b) and finally, histopathological changes in the hematopoietic organs are also evaluated (Szarek et al. 1999b, 2004, 2013). These methods permit evaluating both natural immunity (based on genetics) and that obtained through stimulation including natural infection. Natural antibodies can form without the participation of stimuli, while the initial measurment of their level can indicate what the immune potential is. Using these methods in immunological evaluation permits forecasting the potential effects of various xenobiotics and biopreparations and their impact on fish health. It is known, however, that the IS is dependent on many factors that must be taken into consideration when evaluating its function such as the natural maturity and efficiency of the immune system. Such easy access to learning about immune mechanisms permits improving prevention methods since various model studies allow verifying them, especially when it is necessary to learn about these reactions in species that are being introduced to aquaculture or when evaluating their susceptibility to new pathogens.

Natural resistance (characterized by an inborn predisposition) plays very important role in viral infections as it determines the reaction of the body to viruses, e.g., the high natural resistance of brook trout, *Salvelinus fontinalis* (Mitchill), to VHSV infections. Siwicki et al. (2001f) confirm that following experimental infection 48% of rainbow trout died in comparison to only 4% of brook trout. They also report that following infection the interferon level in brook trout was 295 pg ml^{-1}, while that in rainbow trout was barely 25 pg ml^{-1}. Genetic studies on the resistance of fish to infection with KHV also confirm this (Siwicki et al. 2010a). In addition to applying immunoprophylaxis systematically, in intense culture systems a key aspect of preventing disease is to select genetic lines of fish with high defense potential that is appropriate for the degree of culture intensification and to environmental conditions.

Innate and adaptive immunoprophylaxis as a significant element in the prevention and treatment of fish diseases

Stimulating innate immunity using natural and synthetic immunomodulators

Immunoprophylaxis is currently one of the most significant directions in research the aim of which is to limit or eradicate infectious diseases in animals, including fish (Horne and Robertson 1987, Anderson 1992, Van Muiswinkel and Cooper 1992, Galeotti 1998, Evensen 2009, Mehana et al. 2015). It is of key significance in the prevention and treatment of diseases in controlled rearing. Using vaccines and natural or synthetic

preparations that have a stimulatory effect on both innate cellular and humoral defense mechanisms has limited losses caused by disease in fisheries practice. The positive effects of immunoprophylaxis are especially seen in salmonid culture and in species that are new to culture (silurids, sturgeon, pikeperch, pike, eel, tench) (Siwicki et al. 1996b, 2003c, 2004c, 2005a, 2006a, 2010e, 2011d, 2015, Kolman et al. 2000, Jarmołowicz et al. 2012, Kazuń and Siwicki 2013, Kowalska et al. 2015).

Currently, immunoprophylaxis is divided as follows:

- innate – with the aim of stimulating innate cellular and humoral defense mechanisms against commonly occurring pathogenic microorganisms occurring in waters;
- adaptive (vaccines) – directed at stimulating adaptive resistance against specific pathogens.

Immunoprophylaxis using immunomodulators has been applied in pond fish culture for many years. In Poland, it began to be introduced in the 1980s (Siwicki et al. 1989, 1990b, 1990c). Biological availability, the lack of toxic effects on the bodies of fish and humans, and the lack of any negative impact on the natural environment are the main characteristics of immunomodulators. The division of this group of biopreparations (natural and synthetic) is continually being modified as the search continues for new compounds that would, to the greatest degree, potentially meet the principle tenets of immunomodulation, which are to increase resistance against diseases caused by pathogens (Siwicki et al. 2000b, 2010b, 2012a, Ringo et al. 2012, Mehana et al. 2015).

Several commercial products have been developed for use in aquaculture, i.e., MacroGard, Macrogard-Adjuvant, Aqua Salor, Lomai, Ivostin, Ergosan (Ringo et al. 2012, Mehana et al.2015). Their effectiveness is determined primarily by their biological activeness, but it is also affected by proper application regimes, including the time they are administered (Siwicki et al. 1994a, 1996b, 1998a). Good effects are obtained by administering combined products, e.g., Ergosan (Merck-Shering Plough) as a 0.5% supplement to feed in three cycles for 95 days (Ringo et al. 2012).

To date, many substances have been identified that, through their impact on innate mechanisms, have a protective effect against the consequences of stress stemming from manipulation, chemicals, and therapies and as adjuvants during fish vaccination (Siwicki et al. 2002a, 2003d, 2005b, 2011b, Singh et al. 2010, Jarmołowicz et al. 2013, Kazuń and Siwicki 2013, Mehana et al. 2015). They can be administered through injection, immersion, or orally with the feed. Immunostimulators that are injected once or twice in various doses are primarily treated as adjuvants. This application method is used in intense aquaculture in fish exceeding 10-15 g following anesthetization because the procedure is stressful. Administering these compounds by immersion at doses of 2-10 mg L^{-1} and, most commonly, for 10 min to 1 h, is used in the immunostimulation of fish of up to 5 g. While the stimulation potential of this method is less than that of injection, it is, however, the best mass application method. The most convenient and least stressful way of administering preparations is to add them as supplements to feed in proportions of 0.01 to 4%. While the immunostimulatory potential of the *per os* method is the lowest, and the method requires using large quantities of product and it is the most difficult method to control, it is very useful in the culture of larval fish (Kum and Sekkin 2011). To conclude, immunostimulators can be used in fish culture practice in many different ways. Table 3 presents the most thoroughly studied and best developed immunomodulators in aquaculture.

Natural immunostimulators

Glucans are the most studied group of natural immunomodulators. They are components of cell walls in fungi, algae, and grains. They occur in a wide range of structural forms, i.e., water-soluble oligomers, water-insoluble macromolecules, and as specific compounds. In plants, glucans stimulate the production of antibiotics with low molecular weights known as phytoalexins. In invertebrates, they enhance the activity of polyphenol oxidase, which is an

Table 3
Evaluation of the impact of immunostimulants on immunological parameters in selected species of cultured fish

Immunomodulator	Dose	Administration	Stress reduction and others	Fish species investigated	References
Bioimmuno	0.02% added to feed	3 x daily	under intense rearing conditions	European eel	Siwicki et al. 2015
Leiber® beta-S	200 and 300 g ton^{-1} of feed	for 129 days	fry rearing	common whitefish	Szczepkowska 2009
NuPro *S. cerevisiae*	2-6% feed supplement	for eight weeks	anti-infection immunity	pikeperch	Jarmołowicz et al. 2012, 2013
Levamisole	1-5 mg kg^{-1} depending on fish size	with the feed	increased innate immunity	carp, rainbow trout	Siwicki 1990a, 1990b, 1990c
	up to 5-10 mg L^{-1} 1-5 mg L^{-1}	immersion for 30-60 min. 30-120 min.	increases anti-infection immunity prophylactically and therapeutically	carp	Siwicki et al. 1991
	5-25 ug ml^{-1} medium 6.2-0.8 ug ml^{-1} medium	in vitro	*Y. ruckeri* antigen	carp, rainbow trout	Siwicki et al. 1990a, 1990b, 1990c
	21 or 48 g kg^{-1} feed	with feed for 56 days	increased body mass, body composition	pikeperch	Kowalska et al. 2015
Dimer lysozyme KLP-602	50 ug kg^{-1} mc	injection	stimulation before, during, and after antigen administration	rainbow trout 100-120 g	Siwicki et al. 1998b
β-hydroxy-β-methylbutyrate (HMB)	from 10 to 50 mg kg^{-1} for four or eight weeks	with the feed in spring and fall	increased anti-infection immunity against furunculosis and yersiniosis, strengthen metabolism and detoxification, reduce stress	rainbow trout, wels catfish, African sharptooth catfish, tench, pikeperch	Siwicki et al. 2000c, 2001b, 2003c, 2004e, 2005a, 2006a, 2011c Terech-Majewska et al. 2014c
Macrogard	0.2 g 100^{-1}	in the feed for seven days	protection against *A. salmonicida* infection	rainbow trout	Siwicki et al. 1994a
	0.5% feed	in the feed for seven days	strengthening immunogenicity of the vaccination against *Y. ruckeri*	rainbow trout 90-100 g	Siwicki et al. 2004b, 2004c
	1-2 g kg^{-1} feed	in the feed for six weeks	rearing parameters and innate immunity	pikeperch 12 g	Siwicki et al. 2008a
	0.5; 1.0; 2.0 g kg^{-1} feed	in the feed for one month	anti-infection immunity against *A. hydrophila*	tench	Siwicki et al. 2010e

enzyme responsible for catalyzing the oxidation processes in the hemolymph, while in lower and higher vertebrates, these compounds enhance mechanisms that counteract cancers and activate innate defense mechanisms and antibacterial as well as antiviral immunity (Ingram 1980, Siwicki et al. 2010e, 2015, Bzducha-Wróbel and Błażejak 2011).

Basic research is being conducted on the glucans from Basidiomycota fungi and brewer's yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*). Results obtained from in vitro and in vivo studies show that krestin, lentinan, scleroglucan, and schizophyllan increase immunity in trout and carp against bacterial and viral infections by activating phagocytes and increasing the levels of lysozyme and interferon (Siwicki et al. 2008a, Szczepkowska et al. 2009, Mehana et al. 2015, Meena et al. 2016).

The β-glucans 1,3/1,6 obtained from *S. cerevisiae* are the most active, and their bioavailabilty is the most effective in comparison to that of other glucans tested. Micronized forms of glucan are the most bioavailable since particle sizes from 0.2 to 1 μm can penetrate the intestinal walls more readily and reach the cells of the IS. They demonstrate affinity for four types of immune cell receptors: scavenger receptors (SR); complement receptor 3 (CR3); β GR – dectin 1 receptors; lactosylceramide receptors (LacCer, CD 17), which is important for their activity. β GR – dectin 1 receptors are strictly associated with the expression of cyclooxygenase 2 and is responsible for stimulating the toll-like receptor 2/6 (TLR 2/6). The principle receptor is CR_3, which is why most effects of β-glucan activity are associated with neutrophils, monocytes, and natural killer cells (NK), and, to a lesser extent, with macrophage function (Meena et al. 2016). Other mechanisms of their activity include stimulating immune cells, stimulating the release of cytokines (IL-1, IL-6, IL-8, IL-12, TNF-β), increasing leukocyte and antibody numbers, and stimulating free radical reactions. They are used in highly varied doses, e.g., 0.5-1000 mg kg^{-1} b.w. for 7 d; 0.125 and 0.25 g kg^{-1} feed for 4 to 8 weeks, and even longer (Ringo et al. 2012).

The stimulatory effects of the glucans 1,3-1,6-β-D have been noted in the intense culture of whitefish (Szczepkowska et al. 2009). Statistically significant increases in macrophage phagocytic activity and the proliferation of T- and B-leucocytes stimulated with mitogens were noted in whitefish after they had been fed granulate supplemented with Leiber-Beta S in doses of 200 g and 500 g per ton of feed. Simultaneously, statistically significant increases in lysozyme activity and serum Ig levels were noted. Increased Ig levels after the administration of Leiber-Beta S corresponded with the increased proliferation of B-lymphocytes and had a significant impact on anti-infectious immunity associated with production of innate antibodies. Similar results were observed in carp fry (Siwicki et al. 2012b).

Field studies performed on several species of cultured fish confirm the stimulatory effects of glucans from *S. cerevisiae* on the innate defense mechanisms and anti-infectious immunity (Jarmołowicz et al. 2012, 2013, Siwicki et al. 2012b, Kazuń and Siwicki 2013). The results of experimental and field studies indicate that glucans 1,3/1,6 increases the effectiveness of vaccines against bacterial diseases. Administering glucans with the feed prior to vaccination against yersiniosis, furunculosis, vibriosis, and BKD increases adaptive and innate responses as is manifested in the larger numbers of antibody-producing cells and higher, longer lasting serum antibody titers (Siwicki et al. 2011b).

Synthetic immunostimulators

Levamisole (tetrahydro-2,3,5,6 phenylo-6-imidazothiazole) is used widely in human and veterinary medicine and in aquaculture as an anthelmintic. In the 1980s and 1990s, its immunostimulatory properties in fish were discovered (Anderson et al. 1989, Siwicki et al. 1989, 1990b, Sopińska and Guz 1994, Sopińska et al. 1994). Levamisole increases phagocytosis in macrophages as measured with nitroblue tetrazolium test (NBT), the number of antibody-producing cells, and serum lysozyme activity in carp (Siwicki 1991). Sopińska et al. (1994) examined the immunostimulatory properties of levamisole in carp fry after chronic intoxication with nitrogen

compounds (bath – 1 h in a 5 mg L^{-1} solution). Other tests performed by this team indicate that bathing fish in a levamisole solution (concentration of 10 mg L^{-1} water) and supplementing feed with it for 14 d (5 mg kg^{-1} b.w.) is immunostimulatory and protective as it imparts anti-infection immunity against natural infections (Sopińska and Guz 1994).

Levamisole is considered to be a safe preparation, and even a dose twice that recommended for fish does not adversely affect the immune cells, while a dose four times larger than that recommended did not result in mortality even when administered to hatchlings (Siwicki 1991). Positive effects of levamisole were also noted by Kolman et al. (2000) in their study of its impact on the survival of juvenile sturgeon at critical stages of early rearing, i.e., during the transition period from endogenous to exogenous feeding (between days 6 and 13) and in later stages (between days 17 and 22). This study indicated that the preparation increased fish survival significantly especially when administered prior to the resorption of the yolk sac with 20% higher survival in comparison to that in the control group, while when it was administered after exogenous feeding commenced, the difference in survival was just 6% in comparison with the control group, which did not receive levamisole.

In their study, Gopalakannan and Arul (2006) administered levamisole to carp and Hong Kong catfish (*Clarias fuscus* Lacépède) in doses of 250 or 300 mg kg^{-1} b.w. for 90 d. Improved resistance and better growth rates were noted. Singh et al. (2010) administered the preparation to carp a dose of 250 mg kg^{-1} feed in ponds for 45 d. Higher numbers or levels of leukocytes, erythrocytes, and total protein as well as greater body growth were noted. Following experimental infection with *A. hydrophila*, survival after 30 and 45 d was higher than that noted in the control group. Aly et al. (2010) reported a distinct increase in vaccine efficacy against *A. hydrophila* in carp. Stimulating resistance and increasing survival after experimental infection is noted in groups of fish that were administered levamisole along with vaccination and for the subsequent two months at a dosage of 150 mg kg^{-1} feed. Kowalska et al. (2016a and 2016b) performed studies on pikeperch in which their feed was supplemented with levamisole at a dose of 300 mg kg^{-1} feed for 8 weeks, which significantly increased the cellular response and humoral resistance of the fish while also increasing hematological and biochemical indicator levels in the blood. No negative impact on proximate body composition was noted, which is one of the additional advantages of using this preparation in the culture of this fish species in RAS.

Dimerized lysozyme (KLP-602) is a highly purified ovalbumin protein that is subjected to dimerization. In nature, it occurs as a monomer in fish mucus, serum, gills, gastrointestinal tract, kidneys, and spleen. Concentrations of it depend on the fish species and the level of stress experienced from manipulation, transport, and water contamination (Kiczka 1994, Rymuszka et al. 2005). Dimerization of the compound lowered toxicity in relation to immune cells and increased it activity. Generally, KLP-602 stimulates *in vitro* immune cell activity, and *in vivo* it increases anti-infection immunity in salmonids, cyprinids, silurids, and sturgeons (Kiczka 1994, Klein et al. 1997, 1999, Kolman et al.1999a, Szarek et al. 2004, Terech-Majewska et al. 2004c). *In vivo* and *in vitro* studies indicate that KLP-602 modulates cellular resistance after it has been impaired by the administration of antibiotics, and it can be used to mitigate xenobiotic immunosuppression in fish (Studnicka et al. 2000, Siwicki et al. 2000d, Rymuszka and Siwicki 2003, Rymuszka et al. 2005, Terech-Majewska and Siwicki 2006).

An initial *in vitro* study by Klein et al. (1999) of the compound shows that it stimulates cellular and humoral resistance, while also improves lowered cellular activity and humoral resistance caused by the VHS and IPN viruses. Siwicki et al. (1998f) studied the use of KLP 602 as a prevention measure and in treatment of IPN viral infection and concluded that the compound had a distinct corrective effect on immunological parameters that had been lowered by the impact of the virus when it was administered to naturally infected fish for 7 d at a dose of 10 μg kg^{-1} b.w. The effects were discernible in the fish within 2 to 4 weeks. Higher parameters were noted for RBA, PKA and MTT and humoral parameters (IG) in the

group of infected fish fed feed supplemented with KLP 602 in comparison to those in the IPNV infected as well as those in non-infected control groups. These studies demonstrated for the first time that it is possible to use this immunostimulator in the feed to provide immune protection against the development of diseases in infected fish, and cumulative fish mortality after two months of observations was 30% (KLP-602) in comparison to that among the infected fish that had not been immunized (65%). In vivo studies of Siberian sturgeon, *Acipenser baerii* (Brandt), fry that were subjected to a 30 min. bath in a solution of 0.1 mg L^{-1} KLP 602 indicate there is a stimulatory effect on defense mechanisms. The compound increased the level of γ-globulin (30% over a cycle of 8 weeks), lysozyme (40% for 2 weeks), ceruloplasmin (40% from weeks 3 to 5) in comparison to the levels in the control group (Kolman et al. 1999a). Additionally, supplementation of this preparation to the feed (1g kg^{-1} feed) of Siberian sturgeon with a body weight of 189.7g (+/- 27.35 g) stimulated hematopoietic processes and caused increased cell phagocytosis (Kolman 1999b).

β-Hydroxy β-methylbutyrate acid (HMB) is another compound that has been investigated to determine its potential value as an immunostimulator that could be used in aquaculture (Siwicki et al. 2000c, 2004e, 2005a,b, 2006a). HMB is a keto acid produced by the oxidation of the muscle amino acid leucine. HMB supplementation does not harm animals (even at a dose that is 100 times larger than that recommended). It improves condition and muscle structure and shortens recovery periods following exertion. Additionally, it strengthens general resistance indirectly by reducing muscle protein proteolysis and by strengthening cytoplasmic membranes.

For example, *in vitro* studies performed on cells obtained from the fry of rainbow trout, carp, and wels catfish, among other species, with body weights of 50-100 g indicated the stimulatory effect of various concentrations (from 10, 25, 50 to 100 g ml^{-1} medium) of this keto acid on spleen macrophage RBA and PKA and on the proliferative response of lymphocytes stimulated with the mitogens ConA and LPS. An *in vivo* study of the fry of rainbow trout, wels catfish, and pikeperch with body weights of 10-50 g were administered HMB supplements *per os* at doses of 50, 100, and 500 mg kg^{-1} commercial feed for periods of 2, 4, and 8 weeks. Along with HMB supplementation, the fish were experimentally infected with the pathogenic bacteria *A. salmonicida* and *Y. ruckeri*. The results obtained from the in vivo study indicate that the compound stimulates the macrophages and lymphocytes. Increases in the parameters of the innate cellular and humoral responses studied were statistically significant at all doses and for all periods that were observed. Additionally, after experimental infection, HMB in all of the doses treated reduced mortality between 20 and 50% depending on the species and the length for which the supplement was administered (Siwicki et al. 2000c, 2003c). Similar effects were obtained in *in vivo* studies of pikeperch (Siwicki et al. 2005a, 2005b, 2006a).

Bioimmuno I and Bioimmuno II and III (BIO, IFI Olsztyn) are feed supplements that have met with approval in Poland in culture practice as biopreparations that increase fish resistance to diseases caused by viruses, bacteria, fungi, and parasites. These preparations combine glucans and methisoprinol in different proportions. The glucans used in these preparations are extracted from the walls of brewer's yeast, *S. cerevisiae*. Extraction does not damage the 1,3/1,6 β glucan particles which means that they are still active.

These preparations have positive protective effects and enhance the efficiency of the IS against disruptions caused by contamination of the aquatic environment. Administered during antibiotic therapy, they enhance the effect of antibiotics, which increases and hastens the effects of treatment. Administered as a feed supplement, these preparations are absorbed well in the gastrointestinal tract and potentiate intestinal resistance and limit the penetration of pathogenic viruses and bacteria into the body. Experimental studies and implementation show that after the administration of glucans or methisoprinol, which are the active ingredients of BIO, fish weight gain increases (by 20%), feed utilization is improved, losses caused by infectious diseases

decrease (by 30%), and the quantity of chemotherapeutics used is reduced.

A significant stage of the research was to determine the effectiveness of the combination of glucans and methisoprinol in the prevention and treatment of viral diseases. Fish subjected to stress stemming from errors in rearing and feeding are susceptible to viral infections. These factors predispose them to the development of viral diseases by suppressing innate defense mechanisms. Simultaneously, the penetration of viruses into fish bodies induces immunosuppression, which further facilitates viral penetration and reproduction in the host cells (La Patra et al. 1998, Siwicki et al. 2000a, 2000b, 2001c, 2001d, 2001e, 2004d, 2005c, 2008b, Schulz et al. 2015). In human medicine, Isoprinosine® (methisoprinol) enhances cellular and humoral defense mechanisms, and thanks to its ability to damage the genetic codes of viruses, it is also an antiviral substance. Administering glucan and Isoprinosine® simultaneously increases antiviral resistance in rainbow trout and carp, and using them therapeutically after viral diseases (IPN, VHS, IHN) are diagnosed suppresses the development and transmission within populations and limits the development of secondary bacterial infections (Siwicki et al. 2002c, 2003d, 2008b, 2009b). Bioimmuno (I, II, III), which is enriched with methisoprinol, effectively protects fish from viral infections such as spring viremia of carp (SVC), carp nephritis and gill necrosis (CNGN) caused by the koi herpesvirus (KHV), infectious hemorrhagic necrosis (IHN), and infection of channel catfish virus (CCV) (Siwicki et al. 2008c, 2009b, Kazuń and Siwicki 2013).

Microbiological immunomodulators

Elements of the natural environment such as microorganisms have long been utilized in the prevention and treatment of diseases in humans and animals. A wide range of microorganisms play roles in suppressing the development of other microorganisms. The phenomenon of natural competition also occurs in fish on the surface of their bodies and in the gastrointestinal tract. Probiotics and effective microorganisms are also microbiological immunomodulators that are used. A range of preparations with diverse ingredients have been developed for use with fish that provide protection for the functioning of the gastrointestinal tract as well as for overall health (Qi et al. 2009, Demska-Zakeś et al. 2015).

Effective microorganism (EM) technology was developed through the search for the most effective method to protect the natural environment. Professor Teruo Higa of the University of Ryukyus, Okinawa, Japan, pioneered the development of EM. Following many years of work on utilizing microorganisms in bio-fertilizers, Professor Higa announced the composition and name of his Effective Microorganism Technology (EM™) in 1982. He isolated 81 types of microorganisms that can be consumed by people from among about two thousand that are known to be beneficial. The foundation of the system is the universal preparation EM-1, which comprises lactic acid bacteria, photosynthetic bacteria, yeast, actinomycetes, and fermenting fungi. EM technology is known and applied in 120 countries around the world, including Poland, as an alternative to antibiotics and chemical crop protection products, among other uses. The fundamental principles of this technology correspond to those of organic farming, and they are applicable in aquaculture. The technology is an effective tool for correcting and supervising different ecosystems, improving the quality and health of biological systems. EM technology is applied by aquaculturists of warm-blooded animals and fish as it permits increased production (Brzozowski et al. 2013, Terech-Majewska et al. 2015a, 2016a, Tkachenko et al. 2015). This technology is yet another tool that is currently used in general prophylaxis.

EM technology has been used in rainbow trout culture and has proven to have a positive impact on production and to provide protection after experimental infection (Terech-Majewska et al. 2016a). EM-Probiotic (Greenland, Poland) was used in the study, and it was administered as a 2% supplement to the daily feed ration for a period of 30 days in the summer months (July to August). During these months there is a greater risk of disease caused by

conditionally pathogenic microorganisms. The experiment was conducted on fish at the Department of Salmonid Research in Rutki of the Inland Fisheries Institute in Olsztyn. Control and EM groups of fish comprised 200 individuals each with initial body weights of 30 g. Samples of fish were collected three times at monthly intervals (I in August, II in September, III in October). After 30 days of administering the supplement (II sample collection), the mean b.w. of the fish in the control group was 51.75 g while in the EM group it was 52.79 g, which was a 2% increase in fish body weight in the groups that received the EM supplement. This trend continued throughout the experiment, as was confirmed by the measurements done at sample collection III, when the mean b.w. in the control group was 83.1 g, while that in the EM group was 91.16 g, which was a 9.7% difference in favor of the groups that received the EM supplement. Blood samples taken at the II and III samplings exhibited differing blood serum parameters between the two groups. Innate humoral resistance indicators were higher in the EM group; however, only increases of ceruloplasmin levels following 30 days of supplementation were statistically significant ($P < 0.01$). After sampling II, groups of fish were separated for experimental infection (control and EM groups), and after 14 days of adapting to the new conditions in a closed recalculating system (Faculty of Veterinary Medicine, UWM in Olsztyn), the fish were subjected to experimental infection with *A. salmonicida* (intraperitoneal injection of 0.2 ml bacterial suspension at a concentration of $1x\ 10^5$ cfu ml^{-1}). Mortality stemming from the stressors of transportation and adaptation in the control group was 20%, while in the EM group it was 15%. The control group fish exhibited symptoms of stress such as skin darkening, swimming just under the water surface, and microscopic tests revealed necrosis in the gills. The fish from the EM group exhibited no clinical symptoms signaling any disruption and no changes in behavior. Mortality in the Cinf group (control infected) was 50%, while the EMinf (EM infected) group exposed to *A. salmonicida* mortality was 30%. In the groups that were not infected, Cninf (control not infected) and EMninf (EM not infected), no mortality was noted (Terech-Majewska et al. 2016a).

According to the recommendations of the manufacturer, the initial procedure for administering preparations in the aquatic environment is to add 1 l EM-a (basic preparation) or EM-1 (basic preparation) to 10 m^3 water and administering it 4-8 times per season, and then every 6 weeks until the desired effect is achieved. Fish can also be fed EM bokashi (basic preparation) or be in water to which EM has been added. The effect is high quality meat and improved growth (Mau 2002). The results of EM are clear in fish culture, but the advantages of these preparations can only be confirmed through further studies and developing detailed guidelines for their use (Qi et al. 2009, Rapatsa and Moyo 2013).

Stimulating adaptive resistance with vaccines and auto-vaccines

Adaptive immunoprophylaxis methods are based on knowledge of the creation of immune memory and reactions to repeated penetrations of microorganisms, the memory of which is created by applying vaccines (Van Muiswinkel and Cooper 1992, Toranzo et al. 2009, Van Muiswinkel and Nakano 2014). Searching for effective methods of preventing bacterial diseases superseded the introduction of antibiotics and sulfonamides. The tasks of both classic and modern vaccines is to effectively stimulate innate and adaptive immune responses to create high levels of long-term protection. Their effectiveness is shaped largely by the immunization method. Vaccination by immersion, bath, or injection is highly stress inducing, which limits the effectiveness of the vaccine. An alternative is to administer vaccines with the feed (*per os*) as this eliminates stress and increases the availability of the vaccinated antigen, and, consequently, the effectiveness of the vaccine. The search continues for new ways to introduce antigens into fish, e.g., subcutaneously or rectally (Nakanishi et al. 2002, Winkelbaum er al. 2015).

Vaccines have made it possible to limit antibiotic use to the minimum in many countries, such as

Norway. The most vaccines are used in the USA, where approximately 30 commercial vaccines are registered; there are 19 in Canada and 13 in Japan. Nineteen pharmaceutical companies worldwide sell vaccines for fish (Brudeseth et al. 2013). The number of commercial vaccines available on the Polish market is very limited. Currently, the only vaccines that are registered and available are for yersiniosis and furunculosis: Yersi-fishvax for salmonids (Fatro), Aqua Vac ERM, Aqua Vac ERM Oral, and Aquavac Relera for trout; AquavacFHM for salmon (Merk-Schering-Plough) (http://dziennikmz.mz.gov.pl/DUM_MZ/2016/39/akt.pdf).

The foundations of immunoprophylaxis are consistency, relevance, and application prior to any threats. Prophylaxis programs should take into consideration any and all situations that could limit the effectiveness of vaccination. Immunomodulators such as Bioimmuno I, II, III – IFI, Ergosan, and Levamisole are proven in this role since they can be administered before or after vaccination (Siwicki et al. 1998a, 1998b, 1998d, 2001b). Protection programs can be based on commercial vaccines or auto-vaccines. Auto-vaccines can be formulated with factors that correspond to the epizootic situation, and they can be updated systematically. It is important at each stage of development that fish are ensured of the possibility of having immunoprophylaxis.

By definition "vaccines" are immunological veterinarian products that are manufactured with pathogens that have been isolated from animals or domesticated animals that are used at a given facility or for the treatment of animals at that same facility (Journal of Laws No. 45 of February 27, 2008 consolidated text of art. 3.1. point. 4, paragraph 6 of the Pharmaceutical Law). In other words, these are biological preparations containing the appropriate antigen or a few antigens obtained from pathogenic organisms (viruses, bacteria, fungi, parasites) that stimulate the defense system. The effect of this is the production of specific antibodies by B-lymphocytes and the creation of adaptive cellular response in which sub-populations of T-lymphocytes (Th, Tc) and macrophages participate. Finally, the effectiveness of vaccines is decided by the quantities of antigens, the choice of the strain used to develop the vaccines, and determining the appropriate vaccination program for a given animal or culture facility.

Non-pathogenic microorganisms that are isolated from healthy fish can also be used to prepare vaccines, and they can be added to vaccine compositions after killing (author's own research). The aim of vaccination is to protect fish from diseases without exposing them to potential infections by fostering adaptive cellular and humoral immune responses and immunological memory. The immunological response guarantees a state of peaceful co-existence between the host (fish) and the germ. After vaccination, natural contact with a pathogen will provoke a strong response in the fish that will limit the reproduction of the pathogen and prevent the occurrence of the disease. The effectiveness of immunization depends on the immunogenicity of antigens in the vaccine, fish condition and health at vaccination, the maturity of the immune system (age and weight of fish), a highly active immune system, the temperature at which the vaccination is administered (this determines the efficiency of the immune response), route of administration, and the dose and timing of vaccination (immersion, *per os*).

Vaccination by intraperitoneal injection is thought to be the most effective. This method is disruptive, expensive, stressful for fish, and the minimum body weight of fish that can be immunized is 20 g. The benefit of this method is the long-term protection it confers of 5 to 12 months (Gould 2005). This is an especially common way to vaccinate Atlantic salmon. In countries with highly automated aquaculture, immunization is automated. One of the oldest methods of immunizing fish is the oral route. Duff was the first to write about this in 1942, and the vaccine described contained a killed pathogenic strain of bacteria (Duff 1942, Van Muiswinkel 2008). Currently, this method is reemerging, and immunizing with this method fulfills the role of vaccinations that extend protection (Gould 2005). Administering vaccines *per os* could be even more effective if the appropriate granulate in which the vaccinated antigen is enclosed is available (Siwicki et al. 2004a).

Immersion is the safest method for fish vaccination. It should be applied with fish of a body weight of 4-10 g, at a water temperature exceeding 10°C for salmonids or above 15°C for cyprinids (Gould 2005). From a practical point of view, this method is the easiest to adapt to various facilities with various degrees of technology. It also permits vaccinating fish during transportation (author's own research). Properly prepared vaccines should comprise attenuated bacterial cells at quantities of 1-5 x 10^9 ml^{-1}, and one liter should be sufficient for bathing 100-200 kg of fish. The vaccine is dissolved in a ratio of 1:10 in water from the facility, turn on oxygenation, and submerge a small quantity of fish for 30-60 s. Attenuated bacterial cells penetrate through the gills (about 90%), skin, and lateral line. A certain quantity of them is swallowed and transported to the intestines. They are caught by macrophages on the surfaces of the gills and then transported to the blood vessels and organs of the immune system (spleen and kidneys). For 10-14 d following immunization, the fish should not be subjected to stress. Immersion is the most frequently used method to immunize rainbow trout fry against furunculosis and yersiniosis (Grudniewska et al. 2010, Example 1). If fish weighing 1-4 g are to be immunized, a booster vaccination is recommended. This method can also be applied in vaccinations of older fish, but vaccinating fish of up to 10 g is the most economical. However if the technology at a given facility does not permit this, immunization can be modified in terms of antigen concentration and the period during which the vaccine is active (Example 3). Additionally, the effectiveness of the immersion vaccination is affected positively when the fish are under general anesthesia. This permits eliminating excessive mucus secretion that prevents vaccination antigens from making contact with the gills and increases the availability of the vaccine (Siwicki et al. 2002b, 2003a, 2010b).

Immunization should evoke the appropriate, protective immune response; however, it is not necessary for obtaining the protection of immunity. Studies indicate that the range of effective vaccines for fish has always been multidirectional. The latest strategies aim at developing new vaccines through:

- attenuation or reduction of the virulence of certain strains of viruses or bacteria with genetic engineering
- construction of new genetic structures containing only selected genes responsible for the formation of specific antigens that induce adaptive immunity
- usage of cytokines as factors that support immune response (Secombes 2008).

Although the development of immunoprophylaxis based on immunization has a long history, vaccinations are not common. One particular problem is the shortage of vaccines for viral diseases. However, research into the development of effective preparations is continual. One example is the research to develop a vaccination against the viral infection KHV of carp in Poland (Siwicki et al. 2008c, 2009a). This research attempted to adapt the live, attenuated KoVax (Israel) vaccine to cultivation on carp koi fin cells (KFC). The basic condition for effective stimulation in this case was temperature. The study was performed on carp fry with a body weight of 10-11 g that were immunized with the immersion method (1 ml vaccine in 10 l water per 1 kg of fry) for 40 min. Groups of fish were held at water temperatures of 16, 18, 20 and 22°C. After 21 days, the fish were subjected to infection with a suspension of the CyHV-3 virus isolated from diseased fish in Poland at a concentration of 5 x 10^2 $TCID_{50}$ ml^{-1} with 0.2 ml intraperitoneally, also at various temperatures. The lowest mortality following experimental infection in comparison to the other groups was in the group of fish immunized at a temperature of 22°C (20%). The results indicate that, in the case of this virus and this fish species, the effectiveness of vaccination was determined by the conditions of immunization and contact with the virus. The highest mortality was noted in the group of fish vaccinated at a temperature of 16°C and infected at 22°C (80%). Immunoprophylaxis against KHV infection under the conditions in Polish carp culture facilities was impossible since obtaining fish that are resistant to the virus, which is most active at high temperatures (18-23°C), would have required completely altering production technology. Since fish with mature IS (fry of a minimum of 10 g) in Polish conditions occur in the natural environment, it is difficult to catch them for

immunization. During the fall and spring harvests it is too cold to immunize, because the optimal temperature for carp oscillates above 15°C. The virus inhibits IS function at temperatures up to 22°C, which, in the case of natural infection, is an additional hurdle to proper immunization. The study indicated that this immunoprophylaxis method, which is proven effective in Israel and other countries where carp culture is conducted in intense systems, is of limited use under Polish conditions.

The most convenient immunization method is to administer vaccines with the feed. This method can be used in industrial-scale fish culture, and it is especially good for administering booster vaccinations. The vaccine antigen, which is enclosed in granulate so as to prevent its digestion in the gastrointestinal tract or inactivation by low pH, reaches the lymph tissues in the duodenum section of the small intestine. Laboratory and field studies by Siwicki et al. (2004a, 2006b) evaluated the effectiveness of yersiniosis and furunculosis vaccines based on strains isolated from clinical cases in Poland that were closed in granulate. The results indicate that these preparations do not have a negative impact on fish condition of health. In none of the experimental setups were clinical changes or anatomopathological lesions noted in the fish examined that would suggest the negative impact of the vaccines tested. Initial immunological tests indicate that the vaccine administered *per os* activated innate defense mechanisms (increased levels of secreted lysozyme and gamma-globulin in the serum). Other studies performed simultaneously to determine the impact the vaccines had on adaptive resistance indicated that as early as 7 days following vaccination the first specific antibodies were noted and titers of them increased gradually in the weeks following vaccination.

Despite great progress in developing modern immunological preparations, it is still important in adaptive immunoprophylaxis in to administer auto-vaccines against yersiniosis and furunculosis that are based on bacterial strains isolated from culture facilities (Siwicki et al. 2001a, 2010b, Kozińska and Pękala 2012, Tkachenko et al. 2015a, 2016). Immersion vaccination requires excellent organization, and it is very laborious. In practice, vaccination can be done during transport when fish are held in solutions with the appropriate concentration of vaccine. The length of time of the vaccination can be manipulated to achieve the best results. This provides an additional opportunity to vaccinate fish in periods when they are larger which is an chance to increase protection especially while vaccinating fish that are immunologically mature (Gould 2005). Depending on technical possibilities, the concentrations used are 1:300, 1:400, 1:500 for 0.5 to 1.5 h (author's own research, unpublished results).

Auto-vaccines are confirmed to be highly effective against conditionally pathogenic microorganisms, i.e., *Aeromonas* spp., *Y. ruckeri*, and *Pseudomonas* spp., in combined preparations and also with immunomodulators as adjuvants or when they are included in immunization programs, e.g., dimerized lysozyme (KLP-602) and β-glucans 1,3/1,6 (Siwicki et al.1998b, 1998d, 2002a, 2004c). The positive effects of dimerized lysozyme are observed when it is administered before or along with the vaccine, while β-glucans 1,3/1,6 does not exert a significant impact on the number of ASC cells or on the number of antibodies. A study by Grudniewska et al. (2010) indicates that they can be used in situations when there is a high risk of disease, and even without specific targeting they permit reducing losses by limiting mortality. The auto-vaccines against furunculosis (containing strains of *A. hydrophila*, *A. sobria*, *A. salmonicida*, and *P. fluorescens*) and yersiniosis (containing strains of *Y. ruckeri*) used in the studies were developed in the Department of Epizootiology, Faculty of Veterinary Medicine, UWM in Olsztyn.

Field observations indicate that the protection programs should encompass the entire stock of fish, including those in the full production cycle (Gould 2005, Grudniewska et al. 2009). If small fish with body weights of 1.0-2.5 g or 5-10 g are vaccinated, then a single vaccination is insufficient; however, administering booster vaccines is sometimes not economically justifiable for many aquaculturists. It is worth bearing in mind that the later fish are vaccinated for the first time, then the longer immunity is conferred. This is especially important when fish are

introduced to microbiologically "challenging" environments. Sometimes the timing of immunization can be manipulated to achieve better results. This provides additional opportunities and possibilities to vaccinate fish in periods when they are larger, and this provides an opportunity to increase protection because immunologically mature fish are being immunized. When planning immunization programs the vaccination periods must be designated that include all the fish at a culture facility since the presence of fish that are not immunized can perpetuate the existence of hazards and render microorganisms more virulent, e.g., *Y. ruckeri* (author's own observations, Example 2).

Diversified protection programs, identification of risk factors, and the development of vaccination programs for culture facilities

The physiological requirements of the different developmental stages of fish determine immune system function. Immunoprophylaxis and all rearing procedures must take into consideration the sensitivities of the species that are associated with fish age and the development of the immune system. Currently, it is understood that protection programs start at the stage of genetic selection and the spawning period. Solutions regarding the physiological requirements of spawners can go in two directions: those for stationary cultured fish spawners and those for spawners of fish species that are wild. Broodstocks are subjected to a range of manipulations annually that aim at directing and accelerate selection and ensure that the best possible progeny with high adaptive potential is obtained. Throughout selection the fish become domesticated, and this can also alter their ability to adapt to controlled conditions. Thanks to progress in reproductive biotechnology, it is possible to create broodstocks of fish spawners that are selected from wild fish with the aim, among other things, of supplementing their genetic pool (Szczepkowski and Szczepkowska 2005, Szczepkowski 2011, Zakęś 2009). However, it is more difficult to speak about protecting the health of wild spawners that are caught only to harvest gametes, but transport is important as is keeping these fish under stress-free conditions, as far as this is possible (Kujawa et al. 2006).

Currently, the reproductive biotechnology of fish species that are common in aquaculture is focusing on methods to obtain eggs, evaluating sperm quality, and implementing cryogenic storage standards (Glogowski et al. 2009). In order to achieve the best results, hormonal stimulation is applied during the spawning period. Obviously, the quality of the eggs obtained, determines their ability to be fertilized, proper embryonic development, and even the progeny at subsequent stages of growth. It is also obvious that the stimulation method used to induce spawning impacts gamete quality at the molecular level, which, in effect, refers to gene expression and the quantity of their products. Any change in environmental conditions can affect the cellular organelles of female gametes and the quality and quantity of accumulated material, especially of maternal mRNA, the amount of which determines egg quality (Ocalewicz 2012). Prophylactic methods can directly impact the duration of hatching, e.g., acidic biocides can reduce carp egg incubation time (Kujawa, personal communication). Anesthetics such as 2-fenoksyetanol and MS-222 are used widely in aquaculture. Propiscin (IFI Olsztyn), the active ingredient of which is etomidate, is used in scientific studies. Anesthetizing fish during transport (including spawners) at concentrations of 0.05-0.1 ml L^{-1} water, and inducing general anesthesia during egg harvesting at concentrations of 1.0 ml L^{-1} are both safe for fish. These procedures permit handling fish for 30 min (Kazuń and Siwicki 2001, 2005). The procedure for anesthetizing fish during harvest, transport, egg stripping, check-ups, and for monitoring studies can safeguard eggs from injury, to which they are susceptible for 30 days prior to spawning. This simplifies harvesting gametes and minimizes the stress spawners experience, which results in higher percentages of fertilized eggs and hatch. Using premedication during spawning helps to maintain the condition and health of the fish in the first month following

spawning. Stress leads to weakened resistance barriers (increased permeability for ions and toxins) and accelerates the degradation of blood morphotic elements, which can lead to weakened resistance. Reducing stress also increases the effectiveness of vaccinations (Siwicki et al. 2002b)

The health of females and males has an immediate impact on the condition and health of progeny, especially in the first weeks of life. Significant differences in condition and immune system efficiency are noted in spawners from different facilities because of different epizootic situations, culture systems, and, above all else, the conditions in which they are kept. Males are more sensitive to stressors during the spawning period than are females. Differences are also confirmed among wild spawners. Male sea trout ascending rivers along the Polish coast developed symptoms of ulceration more quickly than did females (field observations). The analysis of their health status did not supply an unequivocal answer as to the causes (Grudniewska et al. 2011, 2012c, Kazuń et al. 2011). Passing immune potential to progeny via transovarial transmission is a phenomenon that has been confirmed scientifically. Thus, it is possible to induce resistance in the first stage of development by preparing females and males for spawning. The choice of feed, dietary supplementation, and other additional procedures can all be manipulated for spawners in culture facilities (Kowalska et al. 2006, Szczepkowski 2008). With wild fish, the only hope is that after spawning the weakest individuals will be eliminated and will not return to the natural environment. Good husbandry and the application of routine procedures with sea trout eggs, hatchlings, and fry that are obtained from fish threatened with ulcerative dermal necrosis (UDN) appears to be sufficient as no increased losses were noted during rearing in comparison to material from healthy fish. After spawning, fish requirements for vitamins, microelements, and protein increase suddenly for regeneration processes in the body and for repairing immune homeostasis. Replenishing these deficits is the foundation of prophylaxis at this stage (Grudniewska et al. 2006). Administering natural and synthetic immunomodulators via intraperitoneal injection or in the feed promotes reconstruction and regeneration while also indirectly supporting immune system function (Anderson and Siwicki 1994, Siwicki et al. 1996c). Good effects obtained after administering Bioimmuno I in female and male rainbow trout, grayling, and brook trout at a dose of 1 kg per 50 kg feed for a period of 30 days following spawning, when the spawners began feeding after spawning, permitted reducing to 1% the percentage of spawner mortality in the 21-day period following spawning. Simultaneously, it was confirmed that spawners (females and males) which received Bioimmuno I immediately after spawning and for one month before spawning (for 10 days), began to spawn more quickly, while the rearing effects obtained as reflected in the percentage of fertilized eggs and hatchlings in the first 21 days was substantially higher. Administering natural immunostimulants with the addition of a selenium + vitamin E complex via intraperitoneal injection 30 days before spawning and immediately following it shortened the recovery period, limited mortality, and had a significant impact on the percentage of fertilized eggs and the condition of the hatchlings in the subsequent spawning season (Siwicki et al. 1996c). Biostim is a new preparation developed at IFI which can be administered to spawners via injection. It exhibits a strong stimulatory effect on anti-infectious resistance immediately following spawning when fish are in a weakened condition.

Administering Bioimmuno I as a feed supplement for a period of 30 days might seem too long, which is why, among other things, Bioimmuno II was developed. Rainbow trout were administered this preparation in the feed for 14 days at a dose of 1 kg per 100 kg. Feeding began 30 days before the anticipated spawning period. The results of the study confirmed that the supplement had a positive impact on the resistance of the spawners. The increase in myeloperoxidase and lysozyme activities and Ig and interferon levels in female and male rainbow trout that received Bioimmuno II suggests that it can be used to prevent viral diseases during periods with higher incidence rates. Initial studies indicate that

the components of the preparation stimulate anti-viral immunity and hinder replication of the IHNV. Currently, Bioimmuno III is under development with the aim of improving its biological properties. Immunomodulation also enhances immune system function, and it can contribute to strengthening metabolic processes.

Adaptive immunoprophylaxis, which is based on new-generation vaccines, serves to stimulate immunological responses to specific pathogens before natural contact is made and the disease occurs. The possibility of inducing adaptive resistance and then passing it on via transovarian transmission is one of the most important procedures for improving anti-infection immunity in progeny. Simultaneously, positive effects are noted in fish condition and rearing parameters in spawners, while larval survival is higher. Systematic immunization with vaccines against bacterial diseases via immersion or intraperitoneal injection be done while monitoring spawners prior to spawning. The analyses of the results of experimental studies indicate unequivocally that spawners tolerate vaccines very well that do not have a negative impact on the percentage of fertilized eggs or on the condition of the hatchlings. Simultaneously, spawners with high specific antibody titers exhibit a higher resistance to the pathogenic bacteria *A. salmonicida* and *Y. ruckeri*. A high percentage of this targeted, adaptive immunity potential against the two pathogenic bacteria species tested was transmitted to the offspring, which resulted in higher antimicrobial resistance in the hatchlings and fry for three months. This same method can be used to confer immunity against pathogens that occur at a given facility. Auto-vaccines, among other things, that are developed for the needs of culture facilities and other entities that are co-operating serve this purpose (Siwicki et al. 2010b, Grudniewska 2009, 2012a, 2012b). A good example of this is the maintenance of the grayling broodstock that is the result of many years of systematic breeding work performed jointly by the Department of Salmonid Research in Rutki and IFI in Olsztyn. The spawners were selected genetically based on appropriate prophylactic and feeding programs, and they were stimulated immunological through vaccination and the administration of immunomodulators (Grudniewska et al. 2012a, 2012b).

Examples of combined strategies taking into consideration the specificities of culture facilities, fish species, and the management of programs to prevent and treat fish diseases

Example 1

Every year at a facility running moderately intensified production excessive mortality occurred in the period from mid-May to mid-August. The impact of vaccination on rainbow trout fry growth and survival was evaluated using two auto-vaccines developed based on a collection of strains against furunculosis (containing killed strains of the bacteria *A. hydrophila* and *A. salmonicida* at a concentration of 1×10^{10}) and against yersiniosis (containing killed strains of *Y. ruckeri* at a concentration of 1×10^{10}), and these were compared to the group that was not vaccinated (control) (Grudniewska et al. 2010). The study material was reared rainbow trout hatchlings from spring spawning with an average initial weight of 1 g. Water temperature during the duration of the experiment ranged from 11.1 to 19.1°C. The fish were divided into three experimental groups: vaccinated by immersion with the vaccine against furunculosis (F); vaccinated by immersion against yersiniosis (J); control group (K). Vaccinations (duration of 30 to 60 s) were performed by immersion in a solution at a concentration of 1:10 with technological water at a temperature of 12.2°C. Before immersion, the fish were anesthetized in a solution of Propiscin (IFI, Olsztyn) to eliminate stress (Siwicki et al. 2002b, 2003a). After vaccination, fish in quantities of 10000 individuals per group were stocked into rotating concrete basins with a surface area of 9 m^2 and a water volume of 2.5 m^3. The fish were fed by automated feeders in cycles of 10 h daily in

accordance with guidelines for the species (Grudniewska et al. 2010). The experiment was conducted during a period when the risks of the fry contracting disease were high. The experiment was divided into two experimental periods because of differing growth rates. The feed used in the first period was Bio-Optimal Start in sizes of 0.8 and 1.1 mm manufactured by Biomar, and in the second period the feed was Nutra in sizes of 1.5 and 1.9 mm manufactured by Nutreco. The growth rate was evaluated by weighing the fish; the first time was when they had obtained a mean weight of 4 g (June 21) and the second at 22 g (August 17). This permitted correcting the experimental stock in terms of stocking density at 10 kg in each experimental group. Among the fish used in the experiment, mortality from hatching to achieving a weight of 1 g was slight and did not exceed 5%.

About ten days after the beginning of the experiment, the fish began to show signs of disease in all the groups tested and mortality increased and remained steady for about 20 days. Survival in the control group in the first stage was the lowest at 51%. In the second rearing period until August 18, mortality in the control group was limited to individuals only, and survival was significantly higher at 92.7%, but this was the lowest in comparison to the survival of the fish immunized against furunculosis (F) and yersiniosis (J) at 98.3 and 94.5%, respectively. At the end of the experiment the mean body weight of one fry individual from the control group was 23.9 g, and this was slightly higher than the mean weights in vaccinated groups F and J by 1.4 and 1.7 g, respectively. In all of the experimental groups, including the control group, the fish became sick and died, but no clinical sings or anatomopathological lesions were noted that would indicate any toxic effects from the tested vaccines. The fish fed normally, and only during the period of intense mortality, especially in the control group, was feeding diminished, and the fish did not consume their daily feed ration. Differences were confirmed in body weight gain, with the vaccinated fish gaining more weight, which indicated that the vaccines had an effective impact on the fish. This was noted in the greater weight gains and better condition of the fry in these groups. Vaccination against furunculosis was more effective, and the highest survival, good body weight growth, and the lowest feed conversion ratio were obtained, which could indicate that this procedure strengthened the general condition of the fish.

Example 2

This program is for rainbow trout stocks in a mid-sized culture facility operated within the Natura 2000 area. The vaccine was developed using strains isolated from *Y. ruckeri* in 2009-2011. In consideration of the particular type of production and the persistent risk of yersiniosis, immunization was performed on fish with body weights of 50-80 g that were moved between the facilities for rearing fry and commercial-sized fish. Vaccination was done during fish transport, which lasted for about 1 to 1.5 h. Vaccine was added to boxes with volumes of 1300 L at quantities of 1.4-1.6 L suspensions, at a concentration on the scale of MF (Mc Farland) 10 (which corresponds to an number of bacteria of 1×10^9). Each transport box held 200 kg of fish, at a temperature range of 7 to 14°C. In the first period of the study (stage I), one group of fish were not vaccinated because the water temperature was too low, and these fish were the stock for one of the ponds. This resulted in a recurrence of disease that required antibiotics. I the subsequent season (stage II), 100% of the stock was vaccinated, and in 2014 the disease did not reoccur. At this stage, the fish at a temperature of 7°C were also vaccinated. The experiment continues and systematic bacteriological monitoring was performed. In April 2015, monitoring was recommenced on the water, sediments, and fish in the whole stream of the upper course of the river that supplies the culture facility and at the upstream and neighboring facilities. Until May 2015 no *Y. ruckeri* bacteria was isolated. However, during spring monitoring, IPN was confirmed at the neighboring facility. This suggested retesting the facility at which IPN was confirmed. *Y. ruckeri* bacteria were cultured from material collected from healthy fish. Three weeks following these tests, at this facility

covered by a program to prevent yersiniosis, there was a classic outbreak of yersiniosis among the fish that had been vaccinated the previous fall at low temperatures. The fish that had not been vaccinated did not contract the disease, and no strains of *Y. ruckeri* were isolated from them. In summer, the clinical form of the disease (local changes on the jaws, skin darkening) reoccurred two more times in the unvaccinated fish stocking material from 2015. All of the isolates were subjected to molecular analysis to evaluate their pathogenicity. Investigations revealed no evidence of molecular diversity, which could suggest the endemic nature of the diseases on this stretch of river. Simultaneously, this indicates that continuing the protection program is required.

Example 3

The whitefish prophylaxis program is based on auto-vaccines administered by immersion at various stages of development and included the spring, summer, and fall fry period. It also includes health hazard monitoring, preparing the fish for open water conditions, and targeted selection work. Simultaneously, three immunization methods for administering booster vaccines were tested. The vaccines were prepared with strains from the collection at the Department of Epizootiology, Faculty of Veterinary Medicine, UWM in Olsztyn. A multivalent vaccine was prepared comprising formalin-inactivated cells from *A. salmonicida*, *A. sobria*, *A. hydrophila* and *P. fluorescens* in various quantities according to the MF 8 scale. The first stage was conducted on fish with body weights of 1 g (summer fry), and the second on fish with body weights of 14 g (fall fry) (Szczepkowska et al. 2014). Next, in the second stage, before the fish were vaccinated the third time, they were given a challange test (ChT) to assess the immunoprotection induced by the first and second vaccinations. The ChT was conducted in the laboratory at the Department of Epizootiology, Faculty of Veterinary Medicine, UWM in Olsztyn.

The results of the first stage were published by Szczepkowska et al. (2014). The experiment was run at the Department of Sturgeon Fish Breeding, IFI Olsztyn in Pieczarki. The material was obtained from the artificial reproduction of wild spawners from Lake Gaładuś. The eggs were incubated and the hatchlings reared in a hatchery equipped with a RAS at the facility. The first stage was based on tailoring the vaccination procedures to the conditions at the culture facility as well as determining its impact on the main biotechnological indicators. Fish with body weights of 1 g (vaccinated – S-1 and not vaccinated N-1) and 14 g (vaccinated S-14 and not vaccinated N-14), aged 90 and 191 days, were vaccinated without being caught or manipulated in the rearing tanks. The fish were subjected to a bath lasting about 1 h in vaccine solutions of 1:500 (1 L of vaccine in 500 L water) in rearing tanks with volumes of 1 m^3 and with a volume reduced to 0.5 m^3. The temperature during the vaccination of group 1 g was 19.1°C and in group 14 g it was 16.3°C. In the later stage after dividing the fish into three experimental groups, the water temperature for the smaller fish was 20.1°C and for the larger fish it was 14.0°C. The growth results from the first stage showed no statistically significant differences in any of the parameters. The fish were observed for 12 weeks. Fish survival in the first stage was 95.9% in group S-1 and 92.7% in group N-1, while in groups S-14 and N-14 it ranged from 94.5 to 95.0%. During rearing in the tanks at the IFI facility in Pieczarki no statistically significant differences in rearing indicators were detected among the groups, while differences in body weight and survival were statistically significant in the group that was moved experimentally to a culture facility. Fish body weight in group N was 16 g and in group S it was 17 g. Differences in survival during the 44 days of rearing were N 82.4% and S 98.6% (a difference of 16%).

The main experiment was continued during a second rearing (2014). After the second immersion, the fish were moved to new conditions (mean water temperature 16°C, pH 7.8, O2 6.8 mg L^{-1}) where they were exposed to a mixed suspension of live bacterial cells of *A. salmonicida*, *A. hydrophila*, and *A. sobria* on a scale of MF 3.3 and 0.2 ml intraperitoneal injection. The relative survival of the fish in group K (control) was 65%, and in group Sz (vaccinated) it was 85%.

Table 4
Parameters of innate humoral immunity in whitefish after vaccination and after experimental infection

	Vaccination II- immersion		Vaccination III immersion		Challenge test		
Test type	C	Vac II im	C	Vac III im	Cni	Ci	Vac II im
TP (g L^{-1})	37.29±4.04	46.94± 1.57***	47.93±3.8	58.62± 6.81**	54.62±8.06	79.24±3.11***	63.36±4.7
Lyz (mg L^{-1})	6.54±1.57	5.19±1.42	9.02±4.07	5.3±1.42**	9.23±2.73	10.33±3.11	10.14±3.84
Cer (IU)	73.36±7.83	73.66±9.68	52.11±4.54	56.19±4.7	62.63±3.84	66.05±7.03	64.19±7.26
IgY (g L^{-1})	9.24±1.99	10.04±2.92	17.05±2.17	13.43±4.36	15.14±5.01	29.66±3.2***	14.43±3.31
ChT (%)					100	65	85

Abbreviations used in the table: C – control; Vac II and III im – vaccinated II and III immersion; Cni – control not infected; Ci – control infected; TP – total protein; Lyz – Lysozyme; Cer – ceruloplasmin; IgY – Immunoglobulin Y; ChT – challenge test ** P< 0.001; *** P < 0.0001.

Table 5
Parameters of innate humoral immunity in whitefish after vaccination by immersion, injection, or in the feed

	Vaccination III			
Test type	C	Vac III im	Vac inj	Vac in feed
TP (g L^{-1})	47.93±3.8	58.62±6.81**	48.1±8.16	62.09±9.33
Lyz (mg L^{-1})	9.02±4.07	5.3±1.42**	5.88±2.2	5.17±1.19
Cer (IU)	52.11±4.54	56.19±4.7	58.66±7.88	50.03±2.57
IgY (g L^{-1})	17.05±2.17	13.43±4.36	13.43±4.36	20.01±6.05

Abbreviations used in the table: C – control; Vac III im – vaccinated III immersion; Vac inj – vaccinated by injection; Vac in feed – vaccine in feed; TP – total protein; Lyz – Lysozyme; Cer – ceruloplasmin; IgY – Immunoglobulin Y; ChT – challenge test ** P< 0.001.

The differences in the immunological parameters after the second immersion were discernible although statistical significance was confirmed for the TP (g L^{-1}) and was 37.29 in group K and 46.94 in group Sz. The gamma globulin (γ-gl, g L^{-1}) level was 9.24 in group K and 10.04 in group Sz. Statistically significant differences in serum test results on the fish that survived the ChT were noted for total protein (TP) in group Knz (control not infected) at 63.36 g L^{-1}, in group Kz (control infected) at 79.24 g, and in group Szzak (vaccinated infected) at 63.36. The remaining indicators, i.e., lysozyme (Liz. mg L^{-1}) and ceruloplasmin (Cer. IU) were at very similar levels. After the conclusion of the experiment, samples were collected from the fish to assess the protective properties of the vaccines. Only *A. sobria* was isolated from the samples collected for bacteriological tests. However, in the sera of fish following the second immersion the titers of specific antibodies were from 1:20 to 1:40 and referred only to reactions with *A. hydrophila* and *P. fluorescens*. In fish following the third immersion, which was performed on fish with a mean body weight of 80-100 g, differences in innate resistance parameters three months following vaccination also referred mainly to TP and γ-gl. In group KTP it was 47.93 g L^{-1} and in group Sz III immersion it was 58.62. The level of γ-gl was 17.05 g L^{-1} in group K and 13.43 in group Sz III immersion. Decreased levels of Liz. and increased levels of Cer. were confirmed in the experimental group, but the differences were not statistically significant. None of the fish reared at the IFI facility in Pieczarki exhibited any health problems throughout the rearing season, which is confirmation that the strategies applied are appropriate. The results of the immunological parameters obtained are presented in Tables 4 and 5.

Conclusions

Improving methods for preventing and treating fish diseases is an undertaking that is executed on many levels. It depends on the species and is strictly associated with production technology. Generally, it is done in stages, i.e., after developing reproduction biotechniques and the technology for rearing fry at various stages of development, prophylactic strategies are developed and improved. These procedures should be adequate to hazards and risk factors. Immunomodulation is an effective method, which, depending on the means utilized, permits improving innate or adaptive resistance. Getting the most from a health protection program depends on close co-operation among aquaculturists, veterinarians (both private practice and of govermment veterinary inspectors), diagnostic laboratories, feed manufacturers and distributors, etc. Official guidelines must be followed in the fight against viruses that are on the list for obligatory eradication. In the face of other health threats, we can deploy the prevention procedures that we create together and adapt them to the actual requirements of culture facilities. Analyses of the results of *in vitro* and *in vivo* laboratory studies and data from field observations leads to the conclusion that immunostimulatory methods can lead to increased production and the prevention of diseases, as well as to increased effectiveness in prophylactic immunization.

Programs for the prevention and treatment of fish diseases at culture facilities should be comprehensive. For example, the Complete Protection Program (CPP) at a carp culture facility should include the following procedures:

- pre-season disinfection of ponds, tanks, and all equipment;
- feeding over-wintering fish to improve condition (during mild winters carp deplete their stored energy prematurely);
- using anti-stress measures during fish transport and when pumping water and fish into ponds;
- administering biopreparations (as injections) containing biostimulators, vitamins, and minerals (A, Se with vitamins E and C);
- using anti-stress preparations during controlled spawning to reduce stress and mechanical injury;
- early administration of biostimulators in the feed to all year classes (after catching over-wintering fish, stocking grow-out ponds) to stimulate regeneration and improve condition;
- performing essential prophylactic baths if skin abrasions and/or other injuries or gill and/or skin parasites are noted during monitoring (catches);
- deworming fish if parasites are confirmed in the gastrointestinal tract (prophylactically or therapeutically depending on the parasite species and the timing of administration).

Analogously, immunostimulators should be used in salmonid culture and also with species that are new to aquaculture regardless of the culture system. Every CPP must identify the critical moments during which methods based on immunostimulation or immunization can be implemented. They work then as a corrective factors. Health problems should be treated as disruptions to welfare. It only some pathological processes can be treated (reversed) quickly and only some parasite species eradicated, while the remaining problems should be successfully avoided (Senger et al. 2012).

Given current trends in the production of organic food, including in aquaculture, biological methods for the prevention and treatment of diseases are preferable as they are safe for fish, consumers, and the environment. Continually improving the immunological condition of fish, beginning with spawners and ending with commercial-sized fish, is a method that corresponds with this movement. Improving methods for preventing and treating fish diseases is a system that aims at properly diagnosing, effectively preventing, and resolving problems which could directly or indirectly limit culture and the development of aquaculture. Creating strategies based on the flow of information among aquaculture facilities, veterinarians responsible for monitoring and intervention, and the scientific community can only serve to strengthen and focus this process.

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