The innate immunity of European eel (*Anguilla anguilla*) growing in natural conditions and intensive system of rearing

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Abstract
The intensive rearing of European eels are systematically developing in many countries. The immune system can be exposed to a variety of physical and chemical stressors. The aim of the present study was to determine the innate immunity in European eels growing in natural condition (in rivers) and intensive system of rearing. The analyses of the results showed that the metabolic activity of blood phagocytes, phagocytic ability (RBA) and potential killing activity (PKA) of blood and spleen phagocytes were higher in European eel from natural condition, compared to fish from intensive system of rearing. The similar pattern was observed in proliferative response of blood and pronephros lymphocytes stimulated by mitogens. Also the myeloperoxidase production by blood neutrophils was higher in eels from natural condition. The results of humoral mediated immunity showed that the lysozyme and ceruloplasmine activity in plasma and total Ig levels in serum were higher in eel from natural condition, compared to the fish from intensive culture. Only statistically significant lower levels of total protein in fish from natural condition were observed.

Key words: *European eel, innate immunity, intensive culture, natural environment.*

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Introduction

The European eel is a one of the most important warm water fish cultures in European and Asian countries. This species is euryhaline fish and have complicated life cycle. This life cycle initiate in the spawning area located in the Sargasso Sea for the European eel. After hatching, the larvae go through different stages while migrating to the coast. There, the leptocephalus stage of larva undergoes a further metamorphosis to the glass eel stage and start to migration into rivers, changing from marine to brackish and fresh water. The infection diseases could affect eels at any stage of the life cycle. At this situation, the study of the eel defence mechanisms is of great important for develops new effective methods of prevention infectious diseases. The intensive rearing of European eels are systematically developing in many countries. Successful commercial production of eel species has been facilitated by the intensification of larval and fingerling rearing techniques. With develop of intensive rearing the new infectious diseases are developing in European eel. The immune system is a highly evolved system that functions to provide organisms with the ability to resist pathogenic agents and characterised by two pathways: innate and acquired immunity. The innate immune defence in eels consists of a large number of humoral and cellular factors, which play an important role as the first line of defence against a wide range of infective agent's [1]. Anti-bacterial peptides, proteins and lectins have been described in the skin mucus of different fish species including eel [2, 3]. Lysozyme, which is present in mucosal layers, eggs and blood play an important role in innate immune system in eels [4]. The acute phase response in fish has been defined as the metabolic and physiological change occurring in response to tissue injury or different infections [5]. This important response involves changes in the hepatic, neuroendocrine, haematopoietic and immune systems for re-establish homeostasis in the fish organism. In eel only the C-reactive protein, lectins and ceruloplasmine activity have been described [6-8]. The nonspecific cellular factors present by macrophages and neutrophils have been examined in different species of fish [9, 10], but

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characterisation of the phagocytes and their phagocytic activity has not been performed in European eel [11]. In different fish species some cytokines have been sequenced, however no research has been conducted on the identification and influence of this protein in the European eel immune response. One of the most important innate parameters in fish is pattern recognition proteins (PRPs) or receptors (PRRs). These parameters recognise pathogen associated molecular patterns (PAMPs) associated with microorganisms. Pattern recognition receptors are toll-like receptors (TLRs), which has received some attention in fish research and have been identified in several species.

The results show similar organisation, expression and ligand characteristics as seen in high vertebrates [12]. All the basic features of the acquired immune system are therefore present in fish. The humoral parameter of the acquired system is the immunoglobulins, expressed either as B-lymphocytes receptor or secreted in serum. In fish functional activity possessed tetrameric IgM immunoglobulin only. Other Ig-like molecules have been identificated in some fish species, which many increase the diversity of the B-lymphocyte recognition capacity [13]. Fish IgM can also be present in relatively high levels in fish serum as natural antibodies, which are believed to have PRP properties [14]. In some fish phagocytic activities of B-lymphocytes was presented and indicate a multiple role of fish lymphocyte [15]. The specific cell-mediated cytotoxicity has been identificated in different fish species infected by viruses and intracellular bacteria [16]. However, none of these processes have been described in European eels.

In European eels the proliferative response on two mitogens, ConA and LPS was examined [8].

The innate immune system can be exposed to a variety of physical and chemical stressors. The interactions of xenobiotics with the immune system could finally induced immunosuppression with develop of viral, bacterial or fungal diseases.

The aim of the present study was to determine the innate immunity in European eels growing in natural condition and intensive system of rearing.

Material and methods

The European ells (*Anguilla anguilla*) from natural condition and intensive system of culture were examined. The fish from natural condition was catch from two rivers (Vistula and Odra). The fish from intensive system of rearing were reared in circular plastic tanks during a study period. The tanks were part of a recirculation system equipped with biological and mechanical filters. The water temperature was maintained at a constant level of about 22-24°C. The eels were fed a commercially pelletted dry feed (Skretting – Nutreco Aquaculture Division, France), which met the nutritional requirements for this species. In order to determine the cellular and humoral defence mechanism parameters in natural and intensive culture condition, 40 healthy eels from each group, approximately 50-200 g, were examined. The fish were anaesthetised in Propiscin (IFI, Poland) and peripheral blood was drawn from the caudal vein by Vacutainer system to the heparinized tubes (Vacutainer set – Vacuette Greiner Labortechnik; 50 IU/ml of heparin).

Isolation of leukocytes from blood and organs

The blood leucocytes were isolated by centrifugation at 2000 g for 30 min at 4°C on the Gradisol G gradient (Polfa), washed three time in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2×10^5 cells/ml of medium. Viability of cells was checked by supravital staining with 0.1% w/v trypan blue (1 : 1 mixture of cell suspension and trypan blue solution). Two hundred cells were counted and only samples containing at least 90% of viable cells were used for experiments. The spleen and pronephros of each fish were removed aseptically and single cells suspension were obtained for isolating individual cells using either a Gradisol (Polfa) or Histopaque-1077 (Sigma) gradients as described by Siwicki and Cossarini-Dunier [17]. To determine the number of viable cells from the pronephros or spleen, the cells were stained with trypan blue (Sigma) and then counted after three washings with culture medium RPMI-1640 containing L-glutamine (Sigma).

Chemicals and assay procedures

For the measurement of intracellular O_2, nitroblue tetrazolium (NBT, Sigma) was used at a concentration of 2 mg NBT/ml of RPMI-1640 medium containing L-glutamine (Sigma). A stock solution of phorbol myristate acetate (PMA, Sigma) was used which contained 50 μg PMA/ml in ethanol and was stored at –20°C until used. For the respiratory burst activity (RBA) assay, 1 ml of PMA stock solution was added to 49 ml of RPMI-1640 medium for the final concentration of 1 μg PMA/ml of medium. Concanavalin A (ConA, Sigma) and lipopolisaccharide (LPS, Sigma) were used as mitogens for the lymphocyte proliferation tests. Tetrazolium bromide of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] (MTT, Sigma) was dissolved in PBS at a concentration of 5 mg MTT/ml and sterilized by filtration. This stock solution was used for the MTT assay.

The NBT-spectrophotometric oxidative radical production assay by blood phagocytes was determined, according to the method previously presented by Siwicki and Anderson [18]. One-tenth ml of a 0.2% NBT in PBS solution was added to 0.1 ml of blood in a microtiter plate well and incubation for 30 min. After incubation, 0.05 ml sample was taken from this mixture, added to 1.0 ml N,N-dimethylformamide (Sigma), centrifuged for 5 min at 3000 × g, and read in a spectrophotometer at 540 nm.

The metabolic activity of blood and spleen phagocytes was determined based on the measurement of intracellular

respiratory burst (RBA) after stimulation by PMA (phormol myristate acetate, Sigma), as described by Siwicki *et al.* [19]. The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 1 × 10^6 cells/ml. On 96-well U-shaped microplates 100 µl of isolated blood or spleen leukocytes were mixed with of 100 µl of 0.2% nitro blue tetrazolium (NBT) solution at pH 7.2 and added 1 µl of PMA. After 30 min of incubation at 22°C, the supernatant was removed from each well. The cells pellet was washed with absolute ethanol and than three times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT after incubation with 2M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.

The potential killing activity (PKA) of blood and spleen phagocytic cells was determined according to the method presented by Siwicki *et al.* [19]. On 96-well U-shaped microplates 100 µl of leucocytes from blood or spleen were mixed with 100 µl of 0.2% NBT and added 10 µl of live *Aeromonas hydrophila* (containing 1 × 10^6 bacteria/ml). The mixture was incubated 30 min at 22°C and the supernatant was removed. The cell pellet was washed with absolute ethanol and three times with 70% ethanol and dried at room temperature. This was followed by the addition of 2M KOH and DMSO to each well. The amount of extracted reduced NBT was measured at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.

The proliferative response of the blood and pronephros lymphocytes (LP) stimulated by mitogen concanavalin A (ConA, Sigma) or lipopolysaccharide (LPS, Sigma) were determined by MTT assay previously described by Wagner [20] and adapted for fish species by Siwicki *et al.* [21]. On 96-well culture plates (Costar, USA) 100 µl of blood or pronephros lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100 U/100 µg/ml) were mixed with 100 µl of RPMI 1640 containing mitogens ConA (5 µg/ml) or LPS (20 µg/ml). After 72 h incubation at 22°C without carbon dioxide atmosphere, 50 µl of MTT solution were added into each well and plates were incubated at 22°C for 4 h. After incubation the plates were centrifuged (1400 g, 5 min). Supernatants were removed and 100 µl of DMSO (Sigma) were added into each well and incubated 15 min at room temperature. After incubation the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.

The myeloperoxidase production by blood neutrophils was measured by the cytochemical staining method [18]. Blood smears were prepared on glass slides, air-dried and then fixed with 95% ethanol for 5 min. The slides were immersed in peroxidase indicator reagent with trizmal buffer (Sigma Kit) for 15 min at 22°C. Finally, the slides were washed with PBS and examined on the microscope with colour video camera (CCD Sony, Japan).

The lysozyme activity in plasma was measured by turbidimetric assay [18]. The assay is based upon the lysis of the lysozyme-sensitive Gram positive bacterium *Micrococcus lysodeikticus* (Sigma) which is obtained freeze-dried from major chemical suppliers. A solution of *M. lysodeikticus* in sodium phosphate buffer was mixed with plasma and incubated at temperature 25°C. The absorbance (450 nm) was measured before and after 15 min incubation in sterile plastic tubes. The standard was hen egg white lysozyme (Sigma).

The ceruloplasmine activity in the plasma was determined spectrophotometrically [22] with modified for micromethods in fish [23]. The plasma was incubated in microplates for 15 min in acetate buffer containing 0.2% p-Phenylenediamine (PPD, Sigma). The sodium azide (0.02%) was used to stop the reaction. The ceruloplasmine activity was measured at 540 nm on the microreader (MRX 3 Dynatech).

Analysis of total protein and immunoglobulin (Ig) level in serum was based on the Lowry micro method (Sigma, Diagnostic Kits). The total Ig level was measured using Lowry micro method adapted for fish species by Siwicki and Anderson [18]. This method requires first precipitating the immunoglobulin out of the serum with polyethylene glycol (10 000 kDa).

Statistical analysis

The results from three sets of experiments were pooled. The mean values and standard deviations from pooled experiments were used for comparison between the groups. Statistical significance was evaluated with the use of the Statgraphics 2.1 Win and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon's rank sum test). For all calculations $P < 0.05$ was assumed as significant.

Results and discussion

Fish are therefore the first animal phyla to possess both an innate and adaptive immune system making the very interesting as regards developmental studies of the immune system. The massive increase in aquaculture in recent decades has also put greater emphasis on studies of fish immune system and defence mechanisms against diseases commonly associated with intensive fish rearing. The objective of the present study was to determine the values of the innate cellular and humoral defence mechanisms in European eel, which are bred intensively in recirculation system and in natural condition. This basic examination provides very important information about physiological levels of nonspecific humoral and cellular protection against pathogens in different environmental condition: in recircu-

Table 1. The innate cellular and humoral immune defence in healthy eels from natural condition (Vistula and Odra rivers) and from intensive system of rearing (mean ± SD, *n* = 40)

Immunological parameters	Eels from natural condition of rivers	Eels from intensive system of rearing
Phagocytic ability of blood leucocytes: NBT reduction (mg/ml)	1.17 ±0.15*	0.89 ±0.09
Myeloperoxidase activity of blood neutrophils (total)	97.0 ±2.5*	90.5 ±1.5
Metabolic activity of blood phagocytes: RBA (OD 620 nm)	0.41 ±0.05*	0.35 ±0.04
Metabolic activity of spleen phagocytes: RBA (620 nm)	0.48 ±0.04*	0.40 ±0.03
Potential killing activity of blood phagocytes: PKA (OD 620 nm)	0.37 ±0.05*	0.41 ±0.04
Potential killing activity of spleen phagocytes: PKA (OD 620 nm)	0.43 ±0.04*	0.37 ±0.03
Blood lymphocyte proliferation stimulated by ConA (OD 620 nm)	0.38 ±0.04*	0.32 ±0.03
Pronephros lymphocyte proliferation stimulated by ConA (OD 620 nm)	0.48 ±0.05*	0.41 ±0.03
Blood lymphocyte proliferation stimulated by LPS (OD 620 nm)	0.32 ±0.03*	0.28 ±0.02
Pronephros lymphocyte proliferation stimulated by LPS (OD 620 nm)	0.42 ±0.03*	0.36 ±0.02
Lysozyme activity in plasma (mg/L)	11.8 ±1.4*	9.1 ±1.2
Ceruloplasmine activity in plasma (IU)	65.3 ±3.0*	53.5 ±2.5
Total protein level in serum (g/L)	57.5 ±4.0	63.2 ±3.8*
Total Ig level in serum (g/L)	13.5 ±1.5*	10.2 ±1.4

**statistically significant P < 0.05*

lation system of water and in river. The innate cellular and humoral defence mechanisms in European eel from intensive system of rearing and from natural condition are presented in Table 1. The analyses of the results showed that the NBT reduction by blood phagocytes, phagocytic ability (RBA) and potential killing activity (PKA) of blood and spleen phagocytes were statistically significantly higher ($P < 0.05$) in European eel from natural condition, compared to fish from intensive system of rearing. The similar pattern was observed in proliferative response of blood and pronephros lymphocytes stimulated by mitogens ConA or LPS. The results showed that the proliferative response of lymphocytes was statistically significantly ($P < 0.05$) higher in European eel from river, compared to fish from intensive system of rearing. Also the myeloperoxidase production by blood neutrophils was statistically significantly higher in eel from natural condition, compared to intensive system of rearing (Table 1). The results showed that eels in natural condition has higher cell mediated immunity and suggested that in river they has a solid contact with different pathogens and activate the innate cellular immune response, important line of defence mechanisms and protection against diseases.

The humoral factors of innate defence mechanisms presented by lysozyme and ceruloplasmine activity in plasma and total protein with Ig levels in serum are shown in Table 1. The results indicate that the lysozyme and ceruloplasmine activity in plasma and total Ig levels in serum were statistically significantly higher ($P < 0.05$) in eel from natural condition, compared to the fish from intensive culture. Only statistically significant ($P < 0.05$) lower levels of total protein in fish from natural condition were observed. In this study we present for the first time that innate defence mechanisms are different throughout the growth period of eel in the wild and under culture conditions. The characterisation of phagocytic response of leukocytes has not been performed in any eel species, an important omission [1]. In our study we analysed the phagocytic ability and potential killing activity of blood and spleen leukocytes and results showed that phagocytic responses in eel are similar compared to another fish species [9, 19, 24, 25]. Also the proliferative response of blood and pronephros lymphocytes stimulated by mitogens present a similar levels compared to the other fish species [9, 24, 25]. Esteve-Gassent *et al.* [4] demonstrated that European eel show a strong lysozyme activity before the start of the grow-out phase and after two

months in an intensive farming environment, fish showed a strong reduction in this activity, which further reduced significantly with time. In our study we observed that under culture condition the lysozyme activity was lower compared to eel from the river. Also total Ig levels demonstrated that fish in natural condition has a permanent contact with pathogens and this environmental factors increase the eel humoral immune response against different pathogens.

This basic information regarding innate cellular and humoral defence mechanisms in healthy European eel reared in intensive culture and natural condition given the possibility to monitoring of eel health by immunological parameters. These studies are very important for developing effective methods of infectious diseases prevention and reducing of mortality in eel's culture.

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