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Influence of nematode *Anguillicoloides crassus* infestation on the cellular and humoral innate immunity in European eel (*Anguilla anguilla* L.)

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Abstract

Parasitic invasions are recognized as one of the primary factors responsible for decreasing populations of European eel. The aim of the present study was to determine the influence of infestation with the nematode Anguillicoloides crassus on the innate immunity in European eel (Anguilla anguilla). Anguillicoloides crassus parasitizes the swim bladder of this fish. Levels of the following immunological parameters were measured: spleen phagocyte respiratory burst activity, spleen phagocyte potential killing activity, pronephros lymphocyte proliferation stimulated by concanavaline A or lipopolisaccharide, plasma lysozyme and ceruloplasmin activity, total protein and immunoglobulin (Ig) serum levels. The analyses of the results of humoral and cellular immunity indicate that all studied parameters were statistically significant higher ($p < 0.05$) in non-infested fish compared to the ones with anguillicolosis except for ceruloplasmin level. These data suggest that the A. crassus infestation in European eel is responsible for a decreased immune response what could result in higher susceptibility to other pathogenic conditions.

Key words: *fish, parasite, helminthes, immunological system.*

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Introduction

The European eel (*Anguilla anguilla*) is a catadromous fish with a complicated life cycle. The species migrates over 5000 km to spawning grounds in the Sargasso Sea [1, 2]. The intensive rearing of European eels is systematically developing in many countries. It is one of the most important warm water fish cultures in European and Asian countries. Long-term economic increasing importance, the impact of anthropopressure on the environment, environmental pollution, and diseases have led to a risk of extinction. The increasing economic importance of fish parasitosis for aquaculture and fisheries has enhanced the interest in the defence mechanisms against these infestations. Both innate and adaptive immune responses are mounted by fish to control parasite infestations [3].

Parasitic invasions are recognised as one of the primary factors responsible for decreasing populations of European eel [4]. One of the major diseases of the European eel in open waters is anguillicolosis, which is a parasitosis of many species of eels (*Anguilla* sp.) caused by nematodes of the family *Anguillicolidae*. In the European eel it is caused by *Anguillicoloides crassus* (Kuwahara, Niimi, and Itagaki, 1974) [5]. This parasite was introduced to Europe in the early 1980s, probably with infected Japanese eel imported from Taiwan [6]. A wide range of paratenic and intermediate hosts in the aquatic environment has allowed *A. crassus* to spread very rapidly throughout Europe [7]. It spread across the continent very quickly and today almost completely corresponds to the reach of the geographical incidence of European eel, from North Africa to Scandinavia, with the exception of Iceland [8]. The success of these parasites depends on small fish, snails, tadpoles of frogs, aquatic insects, and newts, acting as paratenic hosts and transmitting the nematode to eels [9, 10].

Anguillicoloides crassus parasitises the swim bladder of eel. Infestations in European eel can lead to deteriorated condition and poor health, and in extreme cases, death [11-13]. Pathological studies of the impact of *A. crassus* on the European eel in the wild and on farms indicate that these fish experience acute inflammation, fibrosis, and severe thickening of the swim bladder wall causing its lumen and size to decrease [12].

Similar to other fish species, the innate immune system in eels comprises a large number of physical, cellular, and

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humoral factors that act as the first line of defence against invading organisms such as viruses, bacteria, and parasites [14, 15]. Few experimental studies have been carried out in fish, and it well established in vivo that macrophages, neutrophils, eosinophils, and lymphocytes can be involved in the host response to nematodes. Many parasites invade and establish in body tissues and fluids where they are able to survive by virtue of a range of adaptations that reduce the efficacy of the immune system. Invasion of, and establishment in, the swim bladder may confer on the parasite freedom from aggressive immunological responses [16].

The aim of the present study was to determine the influence of infestation with the nematode *Anguillicoloides crassus* on the innate immunity in the European eel (*Anguilla anguilla*).

Material and methods

The study involved fish used to restock Szczecin Lagoon in 2011. Fish came from a fish farm involved in rearing eel fry. The average length of the fish was 162 mm with a mean body weight of 5.32 g. The fish were anaesthetised with Propiscin (IFI, Poland) and peripheral blood was drawn from the caudal vein by Vacutainer system into two tubes: heparinised and non-heparinised (Vacutainer set – Vacuette Greiner Labortechnik; 50 IU/ml of heparin), and the fish were then euthanised. The spleen and pronephros of each fish were removed aseptically. After the autopsy and examination of the swim bladder the samples were assigned to the appropriate group. In order to determine the cellular and humoral defence mechanism parameters 20 samples from each group were examined.

The spleen and pronephros of each fish were removed aseptically and single cell suspensions were obtained for isolating individual cells using either Gradisol (Polfa) or Histopaque-1077 (Sigma) gradients, as described by Siwicki and 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).

The metabolic activity of 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.* [18]. 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 spleen leukocytes were mixed with of 100 µl of 0.2% nitro blue tetrazolium (NBT) solution at pH 7.2, and 1 µl of PMA was added. After 30 minutes of incubation at 22°C the supernatant was removed from each well. The cell pellets were washed with absolute ethanol and then three times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT after incubation with 2 M KOH and DMSO (dimethyl sulfoxide, Sigma) was measured colourimetrically 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 spleen phagocytic cells was determined according to the method presented by Siwicki *et al.* [18]. On 96-well U-shaped microplates 100 µl of leucocytes from spleen were mixed with 100 µl of 0.2% NBT and 10 µl of live *Aeromonas hydrophila* was added (containing 1×10^6 bacteria/ml). The mixture was incubated for 30 minutes 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 2 M 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 pronephros lymphocytes (LP) stimulated by mitogen concanavaline A (ConA, Sigma) or lipopolysaccharide (LPS, Sigma) was determined by MTT assay, previously described by Wagner *et al.* [19] and adapted for fish species by Siwicki *et al.* [20]. On 96-well culture plates (Costar, USA) 100 µl of pronephros lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 m M 2-mercaptoethanol, 1% HEPES buffer, and 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 hours of incubation at 22°C without carbon dioxide atmosphere, 50 µl of MTT solution were added into each well and the plates were incubated at 22°C for 4 hours. After incubation the plates were centrifuged (1400 g, 5 minutes). Supernatants were removed and 100 µl of DMSO (Sigma) were added into each well and incubated for 15 minutes at room temperature. After incubation the solubilised reduced MTT was measured colourimetrically 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 lysozyme activity in plasma was measured by turbidimetric assay [21]. 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 *Micrococcus lysodeikticus* in sodium phosphate buffer was mixed with plasma and incubated at 25°C. The absorbance (450 nm) was measured before and after 15 minutes of incubation in sterile plastic tubes. The standard was hen egg white lysozyme (Sigma).

The ceruloplasmin activity in the plasma was determined spectrophotometrically [22] modified for micro-methods in fish [23]. The plasma was incubated in microplates for 15 minutes in acetate buffer containing 0.2% p-phenylenediamine (PPD, Sigma). Sodium azide (0.02%) was used to stop the reaction. The ceruloplasmin

activity was measured at 540 nm on a microreader (MRX 3 Dynatech).

Analysis of total protein and immunoglobulin (Ig) levels in serum was based on the Lowry micro method (Sigma, Diagnostic Kits). The total Ig level was measured using the Lowry micro method adapted for fish species by Siwicki & Anderson [21]. This method requires first precipitating the immunoglobulin out of the serum with polyethylene glycol (10,000 kDa).

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 Statgraphics 2.1 Win and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon's twin pair analysis). For all calculations $p < 0.05$ was assumed as significant.

Results

Comparison of the innate cellular and humoral defence mechanisms in healthy fish and European eel with anguillicolosis are presented in Table 1. The analyses of the results showed that phagocytic ability (RBA) and PKA of spleen phagocytes were statistically significant higher ($p < 0.05$) in European eel free of nematode compared to the fish with the identified parasites. The similar pattern was observed in proliferative response of blood lymphocytes stimulated by mitogens ConA or LPS. The analyses of the results of humoral immunity indicate that almost all studied parameters were statistically significant higher ($p < 0.05$) in non-infested fish compared to the ones with anguillicolosis. Only statistically significant ($p < 0.05$) lower levels of ceruloplasmin in healthy fish were observed.

Discussion

Fish are the first animal phyla to possess both an innate and an adaptive immune system making them very interesting as regards developmental studies of the immune system. The massive extension 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 influence of infestation with the nematode *A. crassus* on the innate immunity in the European eel (*Anguilla anguilla*). This basic examination provides very important information about physiological levels of nonspecific humoral and cellular protection against pathogens in different health conditions: with and without parasitic infestation.

Nematode infestation exhibits stressors in infected fish and increases susceptibility to stress. Young fish are especially sensitive to stress due to infestation by the L3 stage [24]. The spread of parasite *A. crassus* infestation is very diverse. The extent and intensity of infestation is dependent on water salinity and the age and size of the fish. The nematode was noticed in brackish water and freshwater breeding farms [25-27]. The infestation prevalence in the European eel may be as high as 90-100% with a very high intensity of 30 nematodes in a single fish [28-30]. Such a high infestation level cannot remain harmless to the condition of the fish. A study by Knopf and Lucius (2008) [31] suggests that *A. japonica* as the original host of *A. crassus* is able to mount efficient protective immune responses against its parasite, whereas the newly acquired host seems to lack this ability.

Phagocytosis is one of the main mechanisms involved in the host's protective responses leading to the clearance of pathogens. The analyses of the results showed that the phagocytic ability (RBA) and PKA of spleen phagocytes were statistically significant higher ($p < 0.05$) in fish free of infestation, compared to fish with parasitosis. These results agree with observations by Muńoz *et al.* [32]. Macrophages seem to play an important role in the immune response to helminth parasites in fish. Macrophages and granulocytes around *A. crassus* larvae in the swim bladder wall do not seem to attack the L3 and L4 [33], but it is believed that this cellular response plays an essential role

Table 1. Innate cellular and humoral immune defence in healthy eels and in eels with nematode *Anguillicoloides crassus* infestation (infection) in natural conditions (mean ± SD, $n = 20$; *statistically significant $p < 0.05$)

Immunological parameters:	Group of fish free of nematode	Group of fish with identified nematode
Metabolic activity of spleen phagocytes RBA (OD 620 nm)	0.45 ±0.05*	0.32 ±0.03
Potential killing activity of spleen phagocytes (OD 620 nm)	0.40 ±0.04*	0.29 ±0.04
Pronephros lymphocyte proliferation stimulated by ConA (OD 620 nm)	0.47 ±0.04*	0.34 ±0.04
Pronephros lymphocyte proliferation stimulated by LPS (OD 620 nm)	0.40 ±0.05*	0.27 ±0.03
Lysozyme activity in plasma (mg/l)	12.4 ±1.6*	7.6 ±1.2
Ceruloplasmin activity in plasma (IU)	62.5 ±5.0*	93.5 ±3.5
Total protein level in serum (g/l)	54.5 ±3.0*	41.2 ±4.0
Total Ig level in serum (g/l)	12.6 ±1.4*	9.8 ±1.2

in the development of immunity against *A. crassus* because it results in fibrosis that is thought to inhibit invasion of further juveniles [34]. The macrophage malfunction could be the result of an increase in the stress level, and a direct effect of the parasite itself.

The similar pattern was observed in the proliferative response of 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 without nematode (Table 1). The results showed that infected eels do not have higher cell mediated immunity, which suggests that the presence of the parasite in the swim bladder inhibits the innate cellular immune response, an important line of defence mechanisms and protection against diseases.

The humoral factors of innate defence mechanisms presented by lysozyme and ceruloplasmin activity in plasma, total protein, and Ig levels in serum are shown in Table 1. The results indicate that the lysozyme and total protein and Ig levels in serum were statistically significantly higher ($p < 0.05$) in non-infested eel. This enzyme activity could be dependent on the degree of stress, as well as its intensity and its duration and the type of stressors [35], and the presence of parasites in the swim bladder induces chronic stress in eels. Lysozyme has antiviral, antibacterial, and anti-inflammatory properties, but high lysozyme can result in a significantly higher mortality in fish challenged with pathogens [36, 37].

Only statistically significantly ($p < 0.05$) lower levels of ceruloplasmin (Cp) in healthy fish were observed. Ceruloplasmin is an acute phase protein. Its function is to modulate the immune response. It inhibits bacterial development by depriving it of essential nutrients, i.e. copper ions [38]. Ceruloplasmin is found to be activated by the host immune system during stress conditions. Although high levels of this protein seem to prevent secondary bacterial or viral infections, in this case it is more certainly associated with stress caused by infestation with *A. crassus*.

Serum proteins play an important role in the transport of different substances, defence of the organism against pathological agents, and some other functions. Fish physiological status, age, season, and habitats play a role in serum protein properties. Total protein level and total Ig levels in serum were significantly declined in *A. crassus* infected eels compared with non-infected.

Knowledge of the eel immune system is of importance to maintain good health throughout the grow-out period in eel farms. In summary, our results suggest that *A. crassus* infestation in European eels could be responsible for a decreased immune response, which would result in higher susceptibility to other pathogenic conditions.

The authors declare no conflict of interest.

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