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The seasonal changes in innate immunity of the common carp (*Cyprinus carpio*)

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ABSTRACT

The innate immune response in fishes includes both cellular (phagocytes) and humoral (complement system mainly) components. In fish, as in mammals, reactive oxygen metabolites (ROM) are involved in the respiratory burst of phagocytes and three pathways of complement activation can be discerned. The aim of this study was to analyze the innate immune response of fish using parameters such as respiratory burst of phagocytes and the complement activity of plasma of the common carp (*Cyprinus carpio*, Cyprinidae). Samples from a total of 160 individuals were collected in five periods of the year (early summer, late summer, autumn, winter and spring). Respiratory burst activity of a constant blood volume was measured luminometrically and also calculated per phagocyte number. A trend of negative relation between respiratory burst activity and water temperature was observed, thus the respiratory activity reached the lowest values in summer. Total complement activity of plasma was determined as bacteriolytic activity against bioluminescent bacteria. The highest total complement activity was observed in autumn, it decreased in summer and winter and the lowest activity was detected in spring. The highest activity of alternative pathway of complement activation was detected in spring, which decreased in autumn and the lowest values were found in winter and in summer. To evaluate the effect of steroid hormones, the level of 11-ketotestosterone was analyzed in males and the maximum was found in spring. A negative correlation was found between 11-ketotestosterone and both respiratory burst and total complement activity. Our results indicate that the measured parameters of innate immunity in the common carp are strongly affected by seasonal changes. Moreover, we confirmed that the innate immune response is immuno-suppressed by 11-ketotestosterone in spring.

1. Introduction

The immune system of the teleost fish contains various mechanisms of non-specific (innate or natural) immunity and specific (acquired or adaptive) immunity that determine resistance against pathogenic or parasitic organisms (Du Pasquier, 1993). The non-specific immune response of fish includes cellular (phagocytes) and humoral components (complement system mainly) or the systemic inflammation; specific defence includes cellular (stimulated lymphocytes) and humoral (immunoglobulins) factors (Bols et al., 2001). The immunoglobulins known in fish include mainly IgM, although IgD (Harding et al., 1990; Hirono and Aoki, 2003; Hordvik et al., 1999; Stenvik and Jørgensen, 2000; Wilson et al., 1997) and even IgZ and IgT (Danilova et al., 2005; Hansen et al., 2005; Savan et al., 2005) have also been recently described.

The phagocytes (granulocytes, monocytes or macrophages) are considered to be the non-specific cellular factors of the immune system elaborated against pathogens which overcome the natural barriers. In fish, as in mammals, the stimulation of the phagocyte cell membrane with accompanying activation of the membrane associated NADPH-oxidase initiates increased oxygen consumption and production of reactive oxygen species (ROS) with microbicidal activity in a process termed as the respiratory burst (RB). Production of several ROS, such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$) and the hydroxy radical (OH^-), has been reported in fish (Halliwell and Whiteman, 2004; Tarpey and Fridovich, 2001; Tarpey et al., 2004).

Complement and lytic enzymes (including lysozyme) play an important role of natural defence against pathogens. The complement system is composed of more than 30 individual proteins. In general,

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the fish complement shows a close functional similarity to that of mammals. Antibody dependent classical pathway (CP) and antibody independent alternative pathway (AP) and lectin pathway (LP) were demonstrated at the functional and biochemical levels in some fish species (e.g. Nakao et al., 2006; Nonaka and Smith, 2000). Furthermore, it is known that all pathways of fish complement form structurally and functionally similar terminal membrane attack complexes (MAC) as observed in humans (Nakao et al., 2003, 2006; Nikoskelainen et al., 2002). The complement system participates in proinflammatory, chemotactic and opsonic activities and forms the intersection between non-specific humoral and cellular mechanisms (Ellis, 1999). Further, one of the most important and well known complement functions is the ability to create pores in the cell wall of a pathogen with subsequent killing (Holland and Lambris, 2002). Rubio-Godoy and Tinsley (2004) demonstrated the bacteriolytic activities of fish complement as well as its reactions against some parasites. Generally, non-virulent Gram-negative bacteria are highly susceptible to complement lysis, while virulent Gram-negative or Gram-positive bacteria are less susceptible (Holland and Lambris, 2002).

The immune system in fish is also affected by the level of steroid hormones. The 11-ketotestosterone (11-KT) is a major androgen in the majority of teleost fish, liable for sexual behavior and spermatogenesis with potential immunosuppressive effect, found in higher levels in the blood plasma or serum of males than in females (Borg, 1994). In carp, for example, Watanuki et al. (2002) described the immunosuppressive effect of 11-KT on production of superoxide anion, NO and phagocytosis.

Fish immunity is affected by many parameters in the environment; water temperature being considered as the leading factor. However, the experimental study showed that seasonal effect is a stronger factor than water temperature (Saha et al., 2002). In several reports, lower values of water temperature cause the suppression of acquired immune system measured for instance by lymphocyte activity and antibody production, but innate components of the immune system are relatively independent of temperature (Magnadóttir, 2006). Most of the studies regarding effects of temperature on immune parameters were carried out under controlled conditions (e.g. Alcorn et al., 2002; Nikoskelainen et al., 2004), but there is still a lack of studies investigating the immune system in fish living in natural conditions.

The aim of this study was to analyze some selected parameters of innate immunity in the common carp (*Cyprinus carpio*). We hypothesized that activity of peripheral blood leukocytes and complement activity of plasma are affected by seasonal changes. Moreover, we test the potential immunosuppressive role of 11-ketotestosterone on the immune parameters studied.

2. Material and methods

2.1. Sample collection

A total of 160 three- to four-year-old individuals of the common carp (*C. carpio*, Cyprinidae) (with total body weight 1798 ± 432 g) consisting of 87 males and 73 females were collected from a farmed Vodňany population (University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology in Vodňany; Czech Republic). Sampling was performed in five periods (June 2007 as early summer with water temperature +16 °C; August 2007 as late summer, +18.5 °C; November 2007 as autumn, +4.9 °C; February 2008 as winter, +2.5 °C and April 2008 as spring, +7.5 °C). Spring, early- and late summer represent pre-spawning, spawning and post-spawning periods, respectively, for common carp under Central European climatic conditions. Fish were caught using seine netting, put into 550 l fiberglass tanks filled with pond water and immediately sexed and sampled one by one. A blood sample was obtained using caudal vein punction according to Pravda and Svobodová (2003). The collected blood was mixed with heparin (50 IU.ml^{-1} of blood, Zentiva). Blood samples for respiratory burst activity were analysed within two hours after collection; plasma was separated from the remaining blood and frozen (−80 °C).

2.2. Leukocyte counting

Total leukocyte counts were performed in Bürker's hemocytometer. Heparinized blood was diluted with Natt–Herick solution at 1:200 ratio (Hrubec and Smith, 2000). Differential leukocyte profile was assessed from blood smears stained with Hemacolor set (Merck Co., Darmstadt, Germany). A total of 100 leukocytes were considered and classified using their morphology into three categories: lymphocytes, monocytes and neutrophils. For assessing phagocytic ability of neutrophils, only metamyelocytes or older stages than metamyelocytes are considered to have phagocytic ability. Therefore, the relative count of phagocytes included monocytes, neutrophilic metamyelocytes, bands and segments in our study.

2.3. Respiratory burst activity

Blood samples were prepared for each individual fish according to Kubala et al. (1996), Lundén et al. (2002) and Nikoskelainen et al. (2004). The reaction volume of 250 μl contained 200 μl of diluted blood in Hank's balanced salt solution (5 μl of blood), 25 μl of luminol (Molecular Probes, Eugene, Oregon USA, Leiden, The Netherlands, dissolved in borate buffer, pH = 9, final concentration 10^{-3} mol.l^{-1}) and 25 μl of zymosan particles (Zymosan A from *Saccharomyces cerevisiae*; Sigma, USA, final concentration of 0.25 mg.ml^{-1} reaction mixture, opsonized by incubation with serum from different carps). Generally, this 50× dilution is not sufficiently high for erythrocyte free and plasma protein free measurements of respiratory burst (Lilius and Nuutila, 2006). However, it was used in our study because of lower phagocyte activity in the samples representing late summer. The spontaneous ROS production by whole blood phagocytes was not measured in any period sampled because preliminary experiments showed no phagocytic activity. Thus, samples were activated with opsonized zymozan particles after optimization according to activator concentration, sample volume and blood dilution. The kinetics of luminol-enhanced chemiluminescence (CL) was measured for one hour at room temperature using LM01-T luminometer (Immunotech, Czech Republic). A peak of CL curve (measure in relative light units — RLU) represents the maximal intensity of respiratory burst and its total intensity is defined as reaction curve area — integral (RLU*s).

2.4. Complement activity

Complement activity was measured with modifications according to Virta et al. (1997), Nikoskelainen et al. (2002) and Kilpi et al. (2009). Briefly, the total bacteriolytic activity (TA) including all three pathways or only the alternative pathway was determined using a bioluminescence-based method. We used transformed *E. coli* K12 with luxABCDE gene, originating from soil bacterium *Photorhabdus*, expressing bacterial luciferase (Lux) (Atosuo and Lilius, 2009), this Gram-negative bacterium is very sensitive to complement but not to lysozyme as we checked before. The bacterial luciferase catalyzes the oxidations of a long-chain aldehyde and the reduced flavin mononucleotide ($FMNH_2$) with the emission maxima at 490 nm. Bacteria were exposed at laboratory temperature to plasma obtained from *C. carpio*. Heat inactivation of complement was tested by heating of plasma samples to 44 °C for 20 min (Sakai, 1992) where no killing of *E. coli* under the same conditions as active samples was observed. Plasmid of bacteria contains genes for enzyme luciferase and its substrate — long-chain aldehyde. The light emission of the reaction is positively correlated with the viability of *E. coli* which was measured using