Aquaculture 432 (2014) 46–52

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

The seasonal changes of innate immunity of tench, *Tinca tinca* (L.) with different ploidy level

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ARTICLE INFO

Article history:
Received 24 November 2013
Received in revised form 29 March 2014
Accepted 6 April 2014
Available online 24 April 2014

Keywords:
Tench
Innate immunity
Seasonal changes
Host ploidy status
11-Ketotestosterone

ABSTRACT

The study analysed the effect of fish ploidy level and seasonality on haematological parameters and innate immune response of tench (*Tinca tinca*, Cyprinidae), fish species extensively bred in European aquaculture. Fish samples were collected from a breeding pond in Vodňany (Czech Republic) during four sampling periods (September, December, March and June). All measured haematological parameters (erythrocyte and leukocyte count, haematocrit, haemoglobin concentration) were strongly affected by the season and fish ploidy status. The effect of sex was found only in leukocyte count. Blood innate immunity measured by respiratory burst activity of phagocytes and complement activity differed among seasonal samples. Moreover, respiratory burst activity differed between diploid and triploid tench and the trend of ploidy effect was found also for complement activity. Lysozyme activity in skin mucus was affected only by the season. No effect of sex on innate immunity was observed. The level of stress measured as glucose concentration showed seasonal changes, while no significant difference in glucose between diploid and triploid tench was found. The same trend was observed also for 11-ketotestosterone in males. Our study demonstrated the seasonal variation in haematological and innate immunity parameters of tench and indicates the differences in these parameters between diploid and triploid form of tench.

1. Introduction

The enormous ability of fish to cope with constantly changing environment has been studied for a long time. Fish corporal temperature depends on the environmental temperature, which influences all metabolic processes, including the immune response, reproduction, nutrition, behaviour and level of stress (Bowden et al., 2007; Pedrera et al., 1992). Ambient temperature is considered a critical factor affecting the development of both specific and nonspecific fish immunity (Bly and Clem, 1992). Several studies have previously demonstrated strong seasonal changes of physiological and immune parameters linked to the changes of water temperature in both wild and farmed fish populations (Buchtíková et al., 2011; Lamková et al., 2007; Saha et al., 2002; Swain and Nayak, 2009; Swain et al., 2007; etc.). Generally, low water temperature has an immunosuppressive effect in most fish species (e.g. Bly and Clem, 1992; Nikoskelainen et al., 2004). However, little is known about seasonal changes of health-associated parameters in fish with different ploidy status of natural or artificial origin (e.g. Vetešník et al., 2013). The investigation of such parameters is especially important for aquaculture fish where chromosomal manipulation is often applied to increase growth production (e.g. Benfey and Biron, 2000; Flajšhans et al., 2010; Piferrer et al., 2009).

The components of the innate and adaptive immune systems are commonly divided into physical parameters, cellular and humoral factors, cooperating mutually, and are affected by the endocrine and nervous systems. The non-specific innate part is represented mainly by phagocytes, complement system, antibacterial factors and C-reactive protein while the adaptive part includes stimulated lymphocytes and immunoglobulins (Bols et al., 2001; Ellis, 1999). Non-specific innate immunity in fish with different ploidy status was poorly studied: Budiño et al., 2006; Taylor et al., 2007. The scales, mucous surfaces of skin and gills, and the epidermis act as physical barriers against infection in fish; the mucus layer prevents attachment of bacteria or parasites and contains the substances with antimicrobial activity such as lysozyme, complement components, and proteolytic enzymes (Alvarez-Pellitero, 2008; Magnadóttir, 2006).

Phagocytosis, the cellular ingestion and digestion of particulate matter, is a widely distributed defence reaction occurring in all animal taxa (Pedrera et al., 1992). The phagocytes (granulocytes, monocytes or macrophages) are considered the non-specific cellular factors of the immune system elaborated against pathogens which overcome the natural barriers. The stimulation of the phagocyte cell membrane leads to activation of the membrane associated NADPH-oxidase and initiation

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http://dx.doi.org/10.1016/j.aquaculture.2014.04.006
0044-8486/

of increased oxygen consumption and production of reactive oxygen metabolites (ROM) with microbicidal activity in a process known as the respiratory burst (RB). This process as well as production of several ROM, i.e. the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxy radical (OH^-) have been described in fish (Halliwell and Whiteman, 2004; Tarpey and Fridovich, 2001; Tarpey et al., 2004).

The complement system as an evolutionary novel mechanism of defence in fish plays an important role of natural defence against pathogens, as was studied e.g. in cyprinid (Buchtíková et al., 2011; Collazos et al., 1994a), salmonid (Nikoskelainen et al., 2004) and gadid fish species (Magnadóttir, 2000). The complement in fish is composed of more than 35 soluble plasma proteins and plays an important role in host defence by interacting with components of both innate and adaptive immunity. Its activation products are involved in innate immunity in processes including opsonisation, phagocytosis and cytolysis of pathogens, inflammation, and in mediation and enhancement of humoral immunity (Boshra et al., 2006; Ellis, 2001). 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). The fish complement is analogous to that of mammals i.e. fish complement is initiated by one or a combination of three pathways, the classic (antibody dependent) (CP), alternative (AP) and possibly lectin pathway (LP) (Boshra et al., 2006). Moreover, all pathways can lead in formation of terminal membrane attack complexes (MAC), whose structural and functional similarity to mammalian MAC was described (Nakao et al., 2003; Uemura et al., 1996).

As described above, fish immune system is strongly affected by environmental temperature, but the level of hormones may impact its activity as well. Especially during the spawning period, when the level of sex steroid hormones increases, the ability of fish to defend against parasite or pathogen infection may decrease. The range of immunosuppressive effects is wide, from influencing phagocytic cells to decreasing antibody secreting cells and consequently plasma antibody levels (Cuesta et al., 2007; Hou et al., 1999; Yamaguchi et al., 2001). The effect of sex steroid hormones was also studied among fish with different ploidy level (Felip et al., 2001; Kobayashi et al., 1993; Nakamura et al., 1993).

In our study, tench (*Tinca tinca*), cyprinid fish commonly bred in pond aquaculture, was used. Tench production depends either on natural spawning of selected individuals under controlled conditions, or completely on artificial reproduction (Flajšhans, 1997). While no triploids were detected in naturally reproducing populations yet, high frequencies of spontaneous triploids were found in some tench populations established by artificial propagation (see review by Flajšhans et al., 2010), due to post-ovulatory ageing of yet unfertilised ova. It caused spontaneous diploidisation of the maternal chromosome set which, after fertilisation with a haploid spermatozoon, resulted in production of autotriploid individuals. Hence, tench is also a model for chromosomal manipulations which are a potential tool for production enhancement and/or selective breeding of this species in aquaculture. The aim of the present study was to evaluate the effects of fish ploidy status (diploid versus triploid individuals), seasonality and sex on the selected physiological and immune parameters of tench. We hypothesised that fish seasonality associated with water temperature changes affects directly fish immunity or induces the immunosuppressive effect linked to high energy investment in fish reproduction activity (related to spawning). In addition, glucose concentration as an indicator of fish stress was analysed in diploid and triploid tench.

2. Material and methods

2.1. Sample collection

A total of 160 five- to six-year-old individuals of tench including 81 diploid and 79 triploid individuals were collected from a farmed Vodňany population (University of South Bohemia České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Vodňany, Czech Republic). Sampling was performed in four periods: September 2008; December 2008; March 2009 and June 2009. Spring and summer samples represent pre-spawning and spawning periods for tench under Central European climatic conditions (May–August with pond water temperature ranging at 16–24 °C as seasonal minimum and maximum). Fish were caught using seine netting. Firstly, skin mucus was collected by gently scraping the skin with a scalpel blade and sample was placed into sterile tube. A blood sample was taken from the caudal vein of fish according to Pravda and Svobodová (2003) and 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). The ploidy level was detected using flow cytometry according to Flajšhans et al. (2004). The sex of the fish based on sexual dimorphism was identified and further verified using morphology of gonads. Each individual was measured (S_L — standard lengths in mm). Data of fish investigated including water temperature in each sampling period are shown in Table 1.

2.2. Haematological parameters

Total erythrocyte and leukocyte counts were analysed in Bürker's haemocytometer after dilution of heparinised blood in Natt–Herrick solution (Hrubec and Smith, 2000). Haematocrit was measured in heparinised microcapillaries after 3 min. centrifugation at 15×10^3 rpm, haemoglobin content was analysed photometrically according to Piačková and Flajšhans (2006).

2.3. Respiratory burst activity

Blood samples were prepared for each individual fish according to Buchtíková et al. (2011). Briefly, the reaction mixture contained 50× diluted blood in Hank's balanced salt solution, luminol (Molecular Probes, Eugene, Oregon USA, Leiden, The Netherlands, dissolved in borate buffer, pH = 9, final concentration 10^{-3} mol l^{-1}) and Zymosan A (from *Saccharomyces cerevisiae*; Sigma, USA, final concentration of 0.25 mg ml^{-1} reaction mixture, opsonised by incubation with serum from different tench). The kinetics of luminol-enhanced chemiluminescence (CL) was measured at 22 °C in each of the sampling periods for one hour using LM01-T luminometer (Immunotech, Czech Republic). A peak of CL curve (measured in relative light units — RLU) represents the maximal intensity of respiratory burst.

2.4. Complement activity

Complement activity was measured according to Buchtíková et al. (2011). Briefly, the total bacteriolytic activity (TA) including all pathways was determined using a bioluminescence-based method. The transformed *Escherichia coli* K12 with luxABCDE gene expressing bacterial luciferase (Lux) was used (Atosuo and Lilius, 2009). The bacterial luciferase catalyses the oxidations of long-chain aldehyde and the reduced flavin mononucleotide ($FMNH_2$) with an emission maxima at 490 nm. Bacteria were exposed at laboratory temperature to plasma of tench, as complement source. The light emission of the reaction is positively correlated with the viability of *E. coli* which was measured using LM01-T luminometer (Immunotech, Czech Republic). The time (in hours) required for 50% viability of *E. coli* was evaluated (in triplicates) using kinetic curves corresponding to complement activity of each sample. There is a reciprocal proportion between time of *E. coli* viability and complement activity; the shorter time represents higher complement activity in plasma or serum of identical concentration (500 μl ml^{-1}). For better expression, the complement activity was expressed as inverted values (in $hours^{-1}$) when observed values were