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NON-SPECIFIC DEFENCE MECHANISMS OF RUSSIAN STURGEON (*ACIPENSER GUELDENSTAEDTI* BRANDT) REARED IN CAGES

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ABSTRACT. Studies were performed on healthy Russian sturgeon reared in an open heated water reservoir (near Ostrołęka) from May to October in order to determine non-specific defence reactions. After 1 month adaptation, blood was collected in June, July and October. Temperature changed from 27±2°C in summer to 16±5°C in October. The results showed higher metabolic activity of phagocytes (potential killing activity of about 10.3±2.3 bacterial cells and lisozyme activity of about 95±17 mg/l for June and July, respiratory burst activity - peak in June - of about 0.68±0.07 mg/ml) on the background of higher total counts of granulocytes in sturgeon blood (28.4±5.6%) in warm period. The role of eosinophils in binding free oxygenic radicals is discussed. Ceruloplasmine level in plasma did not change in summer (0.93±0.24 μmol/l) but increased about 42,5 % in the period of temperature downfall. The levels of γ-globulins in the plasma gradually increased (from 6±2.4 to 10.4±3.4 g/l), while total protein content decreased and this correlated with the growth of b.w. (r= -0.9825). Our data suggest that dynamic of non-specific immune mechanisms in Russian sturgeon was caused by the change of the environmental impetus and somatic growth. The study points to the future research needs and directions.

Key words: CHONDROSTEI, NON-SPECIFIC IMMUNITY, PHAGOCYTES, POTENTIAL KILLING ACTIVITY, RESPIRATORY BURST ACTIVITY, LYSOZYME, γ-GLOBULINS, CERULOPLASMIN, LEUCOGRAMME, TOTAL PROTEIN

INTRODUCTION

Sturgeons are ecologically unique species and provide, undoubtedly obvious economic and scientific benefits (Beamesderfer and Farr 1997). Due to this, particular interest was devoted in the last 15 years to the problems of introducing species belonging to the family *Acipenseridae* into European aquaculture (Kolman R. 1966, 1999).

Susceptibility of sturgeons to unfavourable and pathogenetic factors varies considerably (Vikhman 1996; Kolman et al. 1999a), so protection of sturgeon against diseases has become an important issue (Kolman et al. 1998 b, 1999 b,c). However, before the application of immunoprotection it is necessary to understand defence status of this fish in given conditions of rearing. There are only a few data on defence system in sturgeons. These species have some specific anatomical and physical characteristics

(Fange 1986), but they possess the same organ systems that are present in other animals (Ivanova 1995), and are capable of producing antibodies (Lukyanenko 1971, 1989; Partula and Charlemagne 1993; Adkison et al. 1996; Kolman et al. 1999a,b). Administration of bacterial antigens in sturgeon causes the elevation of the antibody titres (both natural hemagglutinins and specific immunoglobulins) coupled with an enhanced γ-globulin content. It also activates phagocytic capacity of leucocytes (Kolman et al. 1999b, c). Ceruloplasmin content increased in sturgeons infested by invasive larvae of *Diplostomum sp.*(Kolman et al.1998c). Non-specific response mediated by phagocytes changed under the influence of infestation as well as under immunomodulating substances (Kolman et. al. 1998 c, d). All of these observations have led us to focus on the formation of these immuno-physiological indices in Russian sturgeon (*A. gueldenstaedti*) reared in the cages.

MATERIALS AND METHODS

Studies were carried out on Russian sturgeon kept in cages placed in a discharge channel from an electric power plant in a fish farm near Ostrołęka. The sturgeons were fed pellets produced commercially by Danish firm Aller-Molle. Studies began following a few weeks of adaptation period, in June, when average weight of the fish was 218±22 g (age 11 months). The fish were in good health and there were no visible cellular and multicellular pathogens. Before blood sample collection, the fish were anaesthetised using *Propiscin* solution. Blood was collected in June, July and October, through cardiac puncture, each time from 10 randomly selected specimen, with a heparinized needle intended for insulin injection, into Eppendorf's test tubes. Plasma was decanted and, after being transported to the laboratory in a cooled box, stored in -20°C until the end of the field experiment. Complete blood samples were processed on the day of withdrawal. Differential leukocyte counts were performed in blood smears stained with May-Grunwald and Giemsa-Romanowski. Determination of respiratory burst activity of leucocytes (RBA) in the blood was based upon the reduction of nitroblue tetrazolium salt, with a spectrophotometric method described by Studnicka et al. (1985). Blood was incubated with an equal volume of 2 % NBT (Sigma) solution for 30 min., after which 50 µl were removed and placed in 1 ml N,N-dimethylformamide (DMF; Sigma), centrifuged for 15 min at 1000 rpm and the absorbance of the supernatant was read at 620 nm. Adhesion of the model *Staphylococcus aureus* 209 P bacteria by polymorphonuclear (PMN) neutrophils was determined in the whole blood

according to the method described by Avtalion and Shahrabani (1975) and O'Neil (1985). Mean counts of bacterial cells absorbed by PMN cells were expressed as PKA (Potential Killing Activity). The bacteriolytic activity of lysozyme in blood plasma (LZM) was determined by a turbidimetric assay based on the lysis of freeze-dried *Micrococcus lysodeikticus* (Sigma) (suspension in phosphate buffer) as described by Studnicka et al. (1986). Hen egg white lysozyme (Sigma) was used as the external standard. The reduction in turbidity was measured for the period of 30 min. From these data, calculations were made of the rate of changes in absorbance at 550 nm during the linear stage of reaction. Lysozyme activity of the samples was calculated using regression analysis from the resulting standard curve and expressed as mg l^{-1} hen egg white lysozyme equivalents. Ceruloplasmine level (Cp) was determined according to the spectrophotometric method described by Rice (1986) with a modification (Siwicki and Andersen 1993). Analysis of total protein level and concentration of circulating total γ-globulins in plasma were based on the biurette colorimetric micromethod. Total γ-globulins were separated from the plasma by precipitation with polyethylenic glycol at average MW of 10,000 (Sigma) and the remaining supernatant was read. This value was subtracted from the total protein to give total γ-globulins. In order to calculate protein content, comparison was performed with the standards, Sigma (Siwicki and Anderson 1993). For statistical analysis, means and standard deviations for all test values were obtained using the Student's t-test. Differences of the means (n=10 fish for each group value) were considered statistically significant at $p < 0.05$ (*) and highly significant at $p< 0.01$ (**).

RESULTS

Temperatures of heated water in the channel did not show dramatic fluctuation and changed from 26° in June and 28° in July, but decreased under seasonal climatic effect to 16° C in October. Fish average body weight at the same time changed as follows: June - 218±22 g, July - 374±35 g (1.72 - fold increase during 1 month), October - 820±78 g (3.76 - fold increase in comparison with June, and 2.19 - fold increase compared with July) (Table 1). High correlation between total protein content in the blood plasma and body weight was confirmed ($r = -0.9825$), and this dependence was described by linear regression (Fig.1): $Y = - 0.032 X + 52.756$, where Y – total protein (g/l), X – body weight (g), for $n = 30$ fishes. Hence, total protein content decreased in

TABLE 1

Mean levels of non-specific immune indices on the background of white blood cell picture, total protein content in the plasma and body weight of Russian sturgeon reared in cages

Indices	21.VI (26^0C) X ± s.d.	21.VII (28^0C) X ± s.d.	21.X (16^0C) X ± s.d.
Average fish body weight (g)	218.0 ± 22.0	369.0 ± 35.0	820.0 ± 78.0
Respiratory burst activity [level of formazan in mg/ml]	0.680 ± 0.072	0.368 ± 0.088*	0.476 ± 0.112*
Potential killing activity [count of model bacterial cells]	9.0 ± 2.0	11.6 ± 2.6	3.9 ± 0.8**
Bacteriolytic lisozyme activity [mg / l]	100.9 ± 24.4	89.1 ± 10.9	59.8 ± 12.3**
Ceruloplasmin [mg/l] [μmol/]	123 ± 25 0.932 ± 0.189	122 ± 38 0.920 ± 0.288	173 ± 60** 1.311 ± 0.454
γ–globulins [g/l]	6.0 ± 2.4	9.1 ± 2.4*	10.4 ± 3.4*
Total protein [g/l]	47.4 ± 7.6	39.0 ± 4.5*	27.3 ± 6.3*
Neutrocytes [%]	19,4 ± 5,0	17,8 ± 9,5	16,8 ± 7,0
Eosinocytes [%]	9.0 ± 1.5	11.2 ± 6.6*	6.2 ± 3.1*
Monocytes [%]	1.0 ± 0.0	2.3 ± 0.8	2 ± 1.0
Lymphocytes [%]	69.5 ± 7.0	68.9 ± 11.3	76.7 ± 7.5

*Explanation: * $p \leq 0.05$, ** $p \leq 0.01$*

this time (from 47.4±7.6 to 27.3±6.3 g/l), while γ - globulin fraction increased (from 6.0±2.4 to 10.4±3.4 g/l). This is the reason for gradual increase, from 12.4 % to 37.8 % ($p<0.01$), of the percentage of γ-globulin level in total protein (Table 1). The γ-globulin fraction content and fish body weight correlated ($r = + 0.8717$). This relation was described by linear regression $Y = 0.006 X + 5.54\,9$, where Y – level of γ - globulins (g/l) and X - body weight (g), for $n = 30$ fishes (Fig. 1).

Monocyte counts decreased significantly, by about 75%, but limphocyte count did not increase in a significant way (Table 1). Total counts of granulocytes did not change in summer (respectively 28.4 % and 29 %), but their counts decreased to 23 % in October. Eosinophils counts were higher in June and July (about 10,6%), but decreased about 45 % in October ($p<0.05$) compared with July (Fig. 2). Neutrophils decreased about 15 % in July and then by another 5 % in October (Fig. 2). The respiratory burst activity (RBA) decreased ($p<0.05$) in July (about 46 %) and October (about 15%) compared with June (Fig. 2). Bacteriolytic activity of lisozyme in plasma also reached a peak in June (above 100±24.4 mg/l), then decreased insignificantly to about

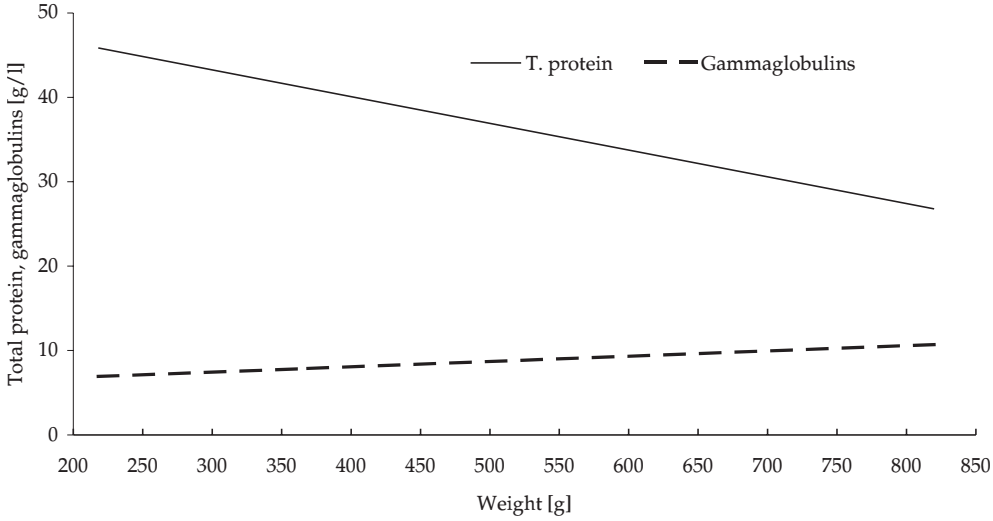

Figure 1. Dependence of total protein and γ-globulins levels on b. w. in Russian sturgeon reared in cages

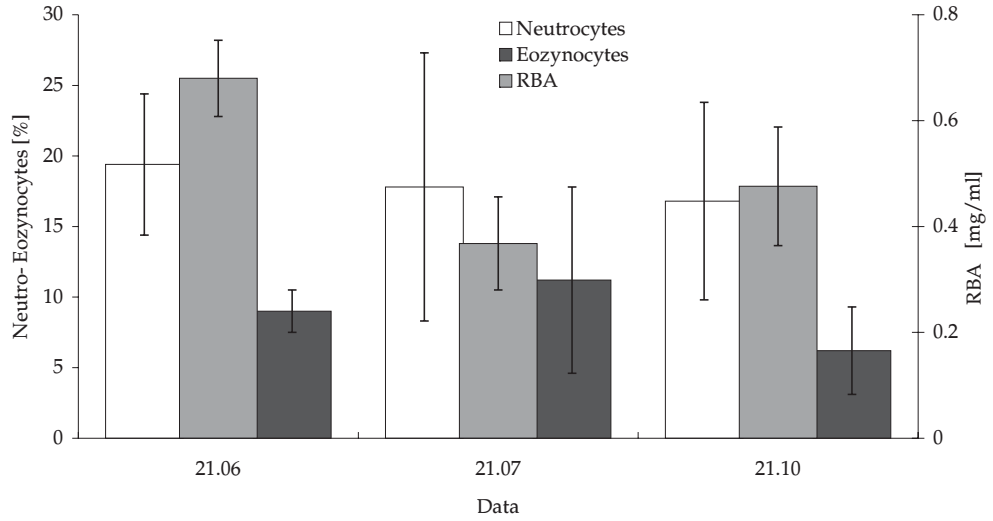

Figure 2. Counts of granulocytes and Respiratory Burst Activity values in blood of sturgeons

90 ±10.9 mg/l (about 12%) in July and to 60±12.3 mg/l (about 33%) ($p<0.01$) in October (Table 1, Fig. 3). Potential killing activity (PKA) of blood PMN cells increased at first not significantly, from June to July (from 9.0 to 11.6), but then decreased to the mean value of 3.9 ($p\leq0.01$) in autumn (Table 1, Fig. 3). The mean content of

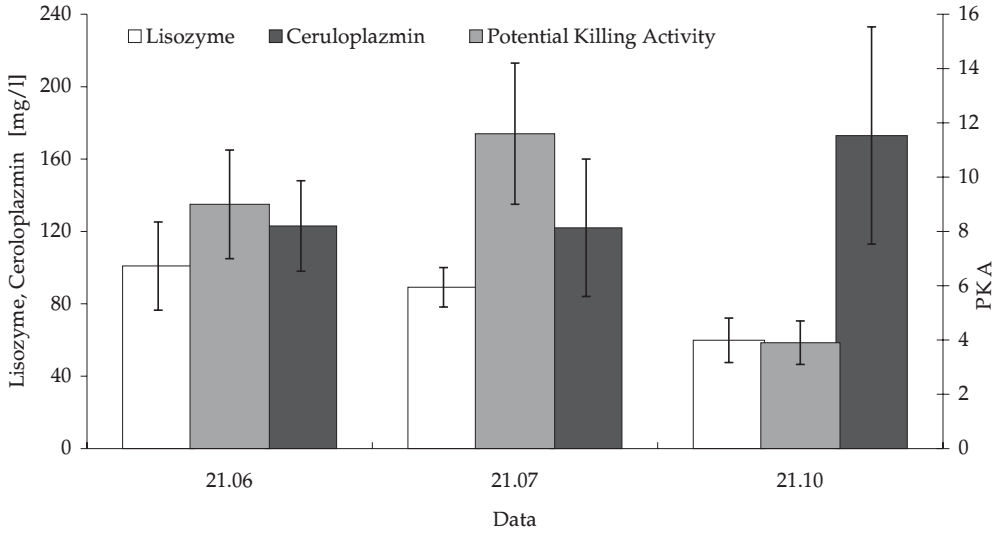

Figure 3. Lisozyme bacteriolytic activity, ceruloplasmin content, Potential Killing Activity in sturgeons reared in cages

ceruloplasmine in plasma did not change in summer samples and remained at the level 0.920±0.288 μmol/l (or in order to compare with LZM level: 123±25 mg/l), but it was higher ($p<0.01$) in autumn, above 42.5 % (1.311±0.454 μmol/l or 173±60 mg/l) compared with summer (Table 1, Fig. 3).

DISCUSSION

Dynamics of total protein content was related to seasonal variations of temperature in the water channel (Table 1). Similar effect of temperature was observed in Russian sturgeon in the Caspian Sea (Lukyanenko 1971, 1989) and in Siberian sturgeon under controlled conditions (Kolman et al. 1998b). Changes of total protein content are affected by feeding intensity and food quality (those factors were constant in this experiment) and strictly related to growth rate and fish metabolism rate (Gershanovich et al. 1987a, b; Kolman R.1999). So, basing on the dynamics of b. w. growth of the studied sturgeons and on total protein content it can be concluded that fish metabolism rate decreased from July to October (Table 1).

Water temperature in the channel decreased in October, although this change proceeded at a slower pace than in natural habitats (Gershanovich et al. 1987a). This might be the reason why displacements in WBC picture in the investigated sturgeons

were less noticeable than in natural Siberian sturgeon population, in which neutrophil counts decreased by 30 % from spring to summer (Yakhimenko 1984). Houston (1990) has questioned whether physiological haematological values can be established for fish species, since such values would depend on the conditions of their environment, but a common trend has been shown in sturgeons (reared in cages) in artificial conditions and in natural habitats: total granulocyte count (Table 1) increased during periods of temperature height and intensive growth rate (Yakhimenko 1984, Gershanovich et al. 1987a, b).

It was shown that potential non-specific (phagocytic) abilities (RBA, PKA, LZM) were activated during high temperatures and intensive growth of sturgeons (Table 1, Fig. 2,3). Oxygen radical production (RBA) increased in June as was determined in Russian sturgeon. Oxygen radicals are produced by macrophages and neutrophils from a membrane-bound NADPH-oxidase enzyme upon phagocytosis (Secombes et al. 1992). *In vivo* the situation becomes fairly complicated due to the fact that functional activation of neutrophils actually contributes to their more compact adherence to blood vessel endothelium, increased migration to the surrounding tissues and inter-tissue spaces, as well as their arresting in the capillaries. The decrease (in July) in the production of these reactive radicals *in vitro* (Table 1, Fig. 2) might be explained by possible appearance of some factors able to uncouple the hexose monophosphate shunt from the production of secretive oxygen radicals in neutrophils (Bomski 1995). But it appears more likely that in sturgeons acidophilic granulocytes will contribute to modulation of potential activities of neutrophilic granulocytes (Kolman et al 1998b), because the eosinophils release the chemotactic factors for neutroplils (Matsuyama and Iida 1999), and produce many electrically charged substances capable of binding free oxygen radicals. Moreover, they possess complement receptors and produce cytokins which stimulate their own functions as well as other leukocyte activities (Rumpley et al. 1999). That is why it seems that eosinophils may play a key role in affecting the amounts of free oxygen radicals (RBA). If so, the examined indices (Fig. 2) might be useful indicators for both sturgeon health and environmental impetus only in their complex analysis (Kolman et al. 1998b).

The role of neutrophils as the source of LZM activity in plasma has been discussed earlier (Alexander 1985; Hine et al. 1986; Kolman et al. 1999b,c) as far as regards the dependence of this enzyme level on temperature (Kolman et al. 1998b). As regards the investigated Russian sturgeon, bacteriolytic lisozyme activity was much higher (Fig. 3) than in fish reared in the recirculation system with complex water purification (Kolman

et al.1998b). This might have been provoked by very high temperature and biotic conditions in the reservoir. It may be assumed that hipersynthesis of lysozyme in the studied fishes depended on the whole phagocytic system functional activity (including tissue macrophages), this being related to mutual relationships between leukocytes and the whole immune recognising and antigen-processing system (Men et al. 1999). This system is related to neuro-hormonal reactions (Ivanova 1995; Vikhman 1996; Weyts et al. 1999), which are also specific for the given open water environment.

It is believed that fish neutrophils undergo phagocytosis in a constitutive way (Avtalion and Shahrabani 1975; O'Neil 1985; Hine et al. 1986; Finco-Kent and Thune 1987; MacArthur and Fletcher 1988), but the mechanisms of this process have not been fully elucidated as yet. In higher vertebrates the share of monomeric immunoglobulin F_c receptors (F_c IgγR) and of the complement components in phagocytosis (Johnson and Smith 1984; Matsuyama et al. 1991; Rose and Levine 1992) are known as well as many other opsonins. In teleost fish, opsonizing role of immunoglobulins is still being verified (Griffin 1983; Sakai 1984; Michel et al. 1990; Koumans-van Diepen 1993). In the case of chondrostom sturgeons there are no data that would describe the opsonizing effects of blood plasma components nor receptors capable of mediating opsonin-independent phagocytosis. It was found, however, that complement activity in this species was subject to seasonal variations: its titre in October was twice higher than in May (Lukyanenko 1971). In view of this, it might be possible that decreased phagocytic ability of PMN cells (PKA) *in vitro* in our autumn samples (Table 1, Fig. 3) was (at least in part) due to the increase of complement bacterioliytic activity towards the used model bacteria (Glowacka et al. 2000).

Fraction of γ-globulins in sturgeon plasma, similarly as in other vertebrates, comprised immunoglobulins (Lukyanenko 1971, 1989; Rudnickaya 1997; Kolman et al. 1999 b, c). Their monomeric molecules possess the amino acid sequences not only like humans, but also similar to teleosts and sharks, and are capable of creating polymers according to the formula: $(H_2L_2)_n$. Pentameric form resembles human IgM (Partula and Charlemagne 1993; Asdkison et al. 1996; Lundqvist et al. 1996, 1998). Seasonal increase of γ-globulins (Fig. 1) has already been observed in Russian sturgeon in the Caspian Sea (Lukyanenko 1971), and ontogenic changes of this index were noted also in other sturgeons (Lukyanenko 1989; Kolman et al. 1998b), but so far it is unconfirmed whether changes in morphology of lymphoidal cells (blastogenesis) and in their number exist in sturgeons.

It was found that ceruloplasmin level in Siberian sturgeon increased by 260 % in course of a 10-fold increment of body weight (Kolman et al. 1998b), and decreased at lower temperatures (in February), when sturgeons stopped feeding, but increased significantly in the period of drop in the temperature (August and September) (Kolman et al. 1999d). Hence, considerable changes in Cp content observed during our study (Table 1, Fig. 3) might have been related to both: fish growth and temperature downfall. The elevation of Cp level was confirmed during parasitic invasion (Kolman et al. 1998c). Hence, ceruloplasmin may act as an acute phase protein in sturgeons in response to abiotic or biotic stress (Kolman et al. 1998c), similarly as it does in other vertebrates under effect of both leucocyte cytokins and neurohormonal stimulation of hepatocytes (Kostro et al. 1996). Moreover, it can be concluded that some humoral factors like γ-globulins and ceruloplasmin (Table 1, Fig. 3), similarly as complement (Lukyanenko 1989), are elevated in sturgeons in response to seasonal decreasing temperatures.

It was shown that the list of the factors examined here might be useful for estimation of non-specific immune status in sturgeons reared in open water reservoirs. Changes of the examined indices were closely related to fish growth and environmental impetus. Study on selected immunological indices in sturgeons during their fattening in heated water also reveales the future research needs and directions in sturgeon immunology.

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STRESZCZENIE

NIESWOISTE MECHANIZMY OBRONNE U JESIOTRA ROSYJSKIEGO (*ACIPENSER GUELDENSTAEDTI* BRANDT), W SADZACH

Badania przeprowadzono na narybku jesiotra rosyjskiego (*Acipenser gueldenstaedti* Brandt), w sadzach (od maja do października) w kanale z pochłodniczą wodą zrzutową z elektrociepłowni, w celu określenia dynamiki nieswoistych reakcji odpornościowych zapewniających homeostazę organizmu u osobników, nie posiadających klinicznych objawów chorób. Jesiotry w wieku 10 miesięcy adaptowano do warunków

panujących w otwartym zbiorniku wodnym w ciągu miesiąca, tzn. od 21 maja do 21 czerwca. Następnie trzykrotnie od nich pobrano krew: w czerwcu, w lipcu i w październiku. Krew pobierano z serca od losowo wybranych 10 jesiotrów w stanie anestezji ogólnej po doskrzelowym podaniu r-ru preparatu Propiscin. W pełnej krwi oznaczano różnicowy obraz krwinek białych, a także wskaźniki charakteryzujące aktywność systemu fagocytarnego, tzn. wybuch tlenowy w krwi (RBA), pochłanianie modelowych bakterii przez komórki polimorfonuklearne (PKA) i poziom bacteriolitycznej aktywności lizozymu (LZM) w osoczu, a ponadto, poziom ceruloplazminy (Cp), frakcji γ - globulinowej i białka całkowitego w osoczu krwi.

Stwierdzono wysoką korelację ($r = -0.9825$) pomiędzy poziomem białka całkowitego a masą ciała u badanych jesiotrów ($p \leq 0.01$). Poziom białka całkowitego stopniowo obniżał się. Masa ciała w ciągu 30 dni chowu od czerwca do lipca wzrosła o 1,72 razy, a w ciągu następnych 90 dni chowu, tj. od lipca do października - o 2.19 razy. Wskazuje to w sposób pośredni na obniżenie intensywności metabolizmu u badanych jesiotrów w drugim okresie.

Zawartość lizozymu (95±17 mg/l), fagocytarna aktywność komórek PMN (10.3±2.3) i poziom ceruloplazminy (0.93±0.24 μmol/l) nie zmieniały się istotnie w lecie przy temperaturze ok. 27°C. W październiku, gdy temperatury spadły o kilkanaście stopni, poziom lizozymu (ok. 40%) i fagocytarny index (ok. 4-krotnie) zmniejszyły się, a poziom Cp wzrósł o 42.5 % ($p \leq 0.05$). Średni poziom γ-globulin stopniowo zwiększał się w korelacji ze zmianami masy ciała ($r = +0.8717$). Wielkość wybuchu tlenowego (RBA) była najwyższa ($p < 0.05$) w czerwcu, tzn. przewyższała średnie wartości w lipcu o ok. 46% i w październiku o ok.15 %. Zmiany wskaźników funkcji fagocytów (RBA, PKA, LZM) zachodziły na tle następujących zmian w różnicowym obrazie białokrwinkowym. Odsetek monocytów i limfocytów nie zmieniał się w sposób istotny. Ogólna suma granulocytów była znacznie wyższa w letnich miesiącach (29%) w porównaniu z październikiem (23%) na skutek wyższej ilości eozynofili w czerwcu i lipcu (śr. 10.6 %) oraz niższej ich zawartości (6.2±3.1 %) w październiku ($p < 0.05$). Dyskutowano wpływ fizjologicznej aktywości eozynofili na wskaźnik RBA.

Otrzymane wyniki sugerują, że dynamika nieswoistych mechanizmów odpornościowych u badanych jesiotrów znajdowała się pod wpływem środowiska i w związku z charakterem ich wzrostu somatycznego. U klinicznie zdrowych jesiotrów w sadzach stwierdzono wyższą aktywność nieswoistych komórkowych mechanizmów w ciepłym okresie (wysokie wskaźniki lizozymu, potencjalnej fagocytarnej aktywności komórek PMN, czyli PKA, wybuchu tlenowego fagocytów, czyli RBA, ogólnej ilości granulocytów) i przeciwnie podwyższenie humoralnych czynników (γ-globulin i ceruloplasminy) wraz ze spadkiem temperatury w październiku. Ponadto w pracy wskazano na przyszłe potrzeby i kierunki badawcze w zakresie immunologii jesiotrów.

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