Resistance in carps (*Cyprinus carpio* L.) affected by a natural bacterial infection

M. STOSIK[1,2], W. DEPTULA[2], M. TRÁVNIČEK[3]

[1]*Higher Pedagogical School, Zielona Góra, Poland*
[2]*University of Szczecin, Szczecin, Poland*
[3]*University of Veterinary Medicine, Košice, Slovak Republic*

ABSTRACT: Sick carps, affected by acute or chronic forms of erythrodermatitis (CE) were studied. The studies were aimed at obtaining pathophysiological data which would permit evaluation of clinical status and prognosis in carps of various age (23 or 28 months). This was approached by estimation of the number of carps and of dynamics of changes in nonspecific humoral and cell mediated resistance and in levels of selected serum proteins. The ingesting index of neutrophilic granulocytes and percent of neutrophilic granulocytes capable of ingesting proved significantly higher in sick carps as compared to the healthy carps, at every age of carps and form of the disease. Metabolic activity of neutrophilic granulocytes, expressed in the percentage of NBT+ cells was the same in carps with the acute CE form and in the control fish. On the other hand, in carps suffering from the chronic form of the disease a significant decrease in the number of cells was disclosed. NBT index and the amount of formazan in fishes with acute CE form demonstrated a decrease as compared to the corresponding values in control carps. Carps with the chronic CE form showed increases in the indices. The level of lysozyme (LZM) was higher in 23 or 28 months old carps with the acute CE form than in healthy fishes. In carps affected by the chronic CE form no significant differences in LZM levels were disclosed as compared to the healthy carps. MPO activity in the sick carps, particularly those with the acute form of CE, was significantly higher than in the control carps. On the other hand, serum globulin and total protein levels in the sick carps were lower than those in the control carps. The two indices showed a particularly extensive decrease in carps affected by acute form of the disease. When all the parameters estimated in fishes with acute CE forms were taken into account, no differences associated with age (23 or 28 months) were observed that could be related to tendencies and directions of the observed changes.

Keywords: neutrophilic granulocytes; lysozyme; myeloperoxidase; serum proteins; sick carps

INTRODUCTION

The growing interest in resistance mechanisms of fishes and cultured fishes in particular results first of all from the hazards of infectious (bacterial, viral) diseases and invasive diseases. The infections and invasions result in disturbed development or death of the fish and, thus, to material losses. Adequate measures of protection of the animals against diseases, particularly against bacterial and viral diseases, represent an important research goal, also from the practical and economic points of view. The most rational solutions within the preventive measures and fish disease prophylaxis should take advantage of the natural protective mechanisms of an organism or immune system (Lamers, 1985; Stosik and Deptuła, 1990; quoted according to Prost, 1991; Van Muiswinkel, 1992; Kodama *et al*., 1993; Myszkowski, 1993). Possibly complete information on the role and importance of individual elements of resistance mechanisms in the fish and fish of various age is important for practical application in increasingly intensive culture of the animals, exposed to a growing scale of hazards.

The present study was aimed at finding out the direction of quantitative indices change and alterations in the activity of selected resistance indices of cell mediated and humoral types and in protein levels in carps of various age, affected by acute or chronic bacterial infection, i.e. by erythrodermatitis.

MATERIAL AND METHODS

The studies were performed on 87 sick carps and 60 healthy carps (Table 1), studied in 6 groups:
– commercial carps, aging 23 months, showing signs of acute erythrodermatitis – group I, or of chronic erythrodermatitis – group III,
controls: healthy carps of the same age – groups II and IV;

– commercial carps, aging 28 months, with signs of acute erythrodermatitis – group V,
controls: healthy carps of the same age – group VI.

Blood of carps was studied. Blood samples were taken from the tail vein to tubes containing heparin (50 IU per ml blood) or to tubes containing no anticoagulants, in field conditions, immediately after taking the fish out of the pond. Laboratory tests were performed 60 to 80 min. after blood sampling, i.e. immediately after delivery of the blood samples to the laboratory.

Non-specific cell-mediated resistance

1. Ability to ingest *Staphylococcus aureus* strain 209P by neutrophilic granulocytes was examined as described by Brzuchowska (1966), as adapted to fishes (Stosik, 1991a). The results were presented as the index of ingestion by neutrophilic granulocytes (Iig) and percent of ingesting neutrophilic granulocytes (%ig).
2. Metabolic activity of neutrophilic granulocytes was determined by:
   a) Nitrotetrazolium blue spontaneous reduction test (NBT), examined by the cytochemical technique of Park *et al*. (1968), as modified by ourselves. The results were presented in percentages of neutrophilic granulocytes which contained deposits of reduced NBT, i.e. formazan (NBT+ cells).
   b) Spontaneous NBT reduction test examined using the microquantitative technique of Raman and Poland (1975) and Sychłowy and Lucas (1978), as adapted to fishes (Siwicki *et al*., 1993) and expressed as NBT index and formazan level (g/l blood).

Non-specific humoral resistance

1. Level of lysozyme (LZM) in the serum established by the plate technique against *Micrococcus luteus,* according to Lie, Syed and Solbu (quoted according to Ellis, 1990).
2. Activity of myeloperoxidase (MPO) of neutrophilic granulocytes, determined cytochemically according to Graham (quoted according to Zawistowski, 1975), as adapted to fishes (Stosik, 1990). The results were expressed as MPO activity index.

Serum proteins

1. Serum globulin level, determined according to Stone and Gitter (1969).
2. Total serum protein, determined by the biuret technique (Stosik, 1996).

Results of the studies were subjected to statistical evaluation using Student's *t*-test for $\alpha = 0.05$. Arithmetical means, standard deviations were calculated and significance of differences between the results obtained for healthy and sick carps was established.

RESULTS AND DISCUSSION

Index of ingestion by granulocytes and percent of ingesting granulocytes (Table 2) were significantly higher in sick carps than in healthy carps, independently of the age of carps and form of the disease. The highest values of the Iig were found in carps with erythrodermatitis in the chronic form. The results are consistent with those of Siwicki *et al.* (1985) performed on 2 years-old carps with branchionecrosis and with the our own earlier studies on 2 years-old (Stosik, 1991b) and 3 years-old (Stosik, 1991a) carps suffering from erythrodermatitis.

Metabolic activity of granulocytes (Table 3), expressed in percent of NBT+ cells, showed no changes in carps with the acute form of the disease as compared to the control fishes. On the other hand, in carps suffering from the chronic form of the disease, a significant decrease in the number of cells was observed. Metabolic activity of granulocytes, expressed by NBT index and formazan level, was lower in carps with the acute disease. The observations confirmed the tendencies and direction of changes noted earlier in 2- or 3-years-old carps (Stosik, 1991b and 1991a, respectively), suffering from erythrodermatitis and in one year-old fishes experimentally infected with bacteria of *Pseudomonas* and *Aeromonas* genus (Siwicki and Studnicka, 1987). In

Table 1. Groups of sick and healthy carps used for the studies

Experimental group	Number of fishes used for the studies	Groups of cultured carps and their symbols	Clinical status	Age (months)	Body weight (g ± 10%)
I	30	commercial carps – K_3	sick – erythrodermatitis, acute form	23	250
II	20	commercial carps – K_3	healthy – control group	23	240
III	27	commercial carps – K_3	sick – erythrodermatitis, chronic form	23	250
IV	20	commercial carps – K_3	healthy – control group	23	250
V	30	commercial carps – K_3	sick – erythrodermatitis, acute forn	28	750
VI	20	commercial carps – K_3	healthy – control group	28	760

carps with the chronic form of erythrodermatitis the change in granulocyte metabolic activity, manifested by an increase in NBT index and in formazan level was consistent with the results of studies conducted on 2 years-old carps with the chronic form of brachione-crosis (Siwicki *et al*., 1985) and in 2- or 3-years-old carps suffering from erythrodermatitis (Stosik, 1991b and 1991a, respectively). Summing up the results of studies on metabolic activity of granulocytes, it should be stressed that the absence of correlation between results obtained by the cytochemical technique (% NBT+ cells) and those obtained in the microquantitative NBT reduction test (NBT index, formazan level) which has been noted at different stages of individual development of the fish, finds confirmation in earlier results obtained in carps (Stosik, 1991a, b) as well as in humans and "higher" animals (Raman and Poland, 1975; Sychłowy and Lucas, 1978; Salwa, 1980). Also the observed in our studies absence of correlation between ingesting ability of granulocytes and metabolic activity of granulocytes in fishes with acute forms of the diseases finds confirmation in earlier studies in carps with bacterial infection (Stosik 1991a, b).

The lysozyme level in carps of different age, affected with an acute disease process (Table 4) was significantly higher than in healthy fishes (Table 4). The results confirm data of Siwicki and Studnicka (1987), who reported similar changes in one year-old carps experimentally infected with *Pseudomonas alcaligenes* and *Aeromonas punctata* bacteria. On the other hand, no changes, as compared to the control carps, have been observed in lysozyme levels in 23 months-old carps suffering from a chronic form of erythrodermatitis, even if a weakly expressed tendency to lowered levels could be observed in such carps. A similar trend of changes in LZM levels has been demonstrated in carps chronically intoxicated with zinc sulphate (Svobodova and Vykusova, 1989) and the in rainbow trout *(Oncorhynchus mykiss)* (Mock and Peters, 1990). The recorded changes in the level and activity of LZM in fishes reflect reactivity of resistance systems in the fishes or alterations in the reactivity. As indicated by studies of Mock and Peters (1990), situations weakly stressing the fish may induce either an increase or a decrease in the level and activity of the enzyme. Interpretation of the findings is difficult, particularly since the mechanism of stress ef-

Table 2. Ingesting abilities of neutrophilic granulocytes in sick and healthy carps

Experimental group	Type and form of the disease	Age (months)	Neutrophilic granulocytes	
			Iig	% ig
I	erythrodermatitis – acute form	23	34.11 ± 6.36**	48.36 ± 4.39**
II	healthy	23	8.11 ± 1.24	13.23 ± 2.36
III	erythrodermatitis – chronic form	23	47.32 ± 10.07**	47.91 ± 4.86**
IV	healthy	23	8.11 ± 1.24	3.23 ± 2.36
V	erythrodermatitis – acute form	28	31.37 ± 7.21**	43.39 ± 5.27**
VI	healthy	28	7.78 ± 1.24	13.07 ± 1.53

Iig = index of ingestion by neutrophilic granulocytes
% ig = % of neutrophilic granulocytes capable of ingestion
**statistically significant increase in the value as compared to the control group

Table 3. Metabolic activity of neutrophilic granulocytes in sick and healthy carps

Experimental group	Type and form of the disease	Age (months)	NBT test		
			% of NBT+ cells	NBT index	amount of formazan (g/l blood)
I	erythrodermatitis – acute form	23	9.37 ± 0.92	0.06 ± 0.01*	1.26 ± 0.23*
II	healthy	23	9.38 ± 0.80	0.25 ± 0.07	1.58 ± 0.23
III	erythrodermatitis – chronic form	23	5.98 ± 1.10*	0.89 ± 0.20**	2.11 ± 0.48**
IV	healthy	23	9.38 ± 0.80	0.25 ± 0.07	1.58 ± 0.23
V	erythrodermatitis – acute form	28	9.56 ± 0.92	0.07 ± 0.02*	1.28 ± 0.17*
VI	healthy	28	9.41 ± 0.90	0.22 ± 0.04	1.57 ± 0.33

*statistically significant decrease in the value as compared to the control group
**statistically significant increase in the value as compared to the control group

fects on the amount and activity of lysozyme in fishes has not been clarified yet.

Activity of myeloperoxidase in carps with acute form of erythrodermatitis (Table 4) was higher than in healthy carps. A similar trend in MPO activity changes was observed in carps affected by the chronic form of the disease; our own earlier observations on two years-old carps in which acute or chronic form of erythrodermatitis was diagnosed (Stosik, 1991a, b) and data of Studnicka and Siwicki (1990) recorded on one year-old carps invaded by *Eimeria subepithelialis*, confirm the importance of non-specific resistance in the species. The higher MPO activity in the sick carps documents the stimulatory effect of bacterial and parasitic factors on neutrophilic granulocytes and indicates that the MPO-hydrogen peroxide system plays a significant role in elimination of pathogenic factors in carps. The presented direction of changes in both indices of non-specific humoral immunity (LZM level and MPO activity) confirms a significant role of non-specific immunity in fishes.

The levels of markers which also indirectly reflect the condition of specific humoral immunity, i.e. the levels of globulin and total protein, have behaved in a distinct manner. The typical decrease in globulin level and in total serum protein, observed in our studies in sick carps, has been independent of fish age and form of the disease (Table 5). It should, however, be stressed that in carps affected by chronic form of erythrodermatitis, the decrease in total globulins, even if significant, has been two- to three-fold less pronounced than in the fishes affected by the acute form of the disease. The results correspond to observations of Svobodova and Vykusova (1989) in carps subjected to chronic intoxication with zinc sulphate in which decreases in globulin level and in total protein were disclosed. Also the studies of Evenberg *et al*. (1986) on *Aeromonas salmonicida* infection affected carps confirmed the trend noted in the present studies and pertaining the discussed indices. It should be added that the reasons for the decreased total protein and the lowered globulins in bacterial infection affected fish are far from being clear (Evenberg *et al*., 1986). The decreases may be linked to increased permeability of blood vessels due to augmented histamine release (Ellis *et al*., 1981), to inhibited protein synthesis and to the lowered or absent appetite in the sick fishes (Evenberg *et al.*, 1986). According to the latter authors, most probably the phenomenon results first of all from non-specific proteolysis of serum proteins, as earlier remarked by Ellis (quoted according to Evenberg *et al*., 1986). The hypothesis was confirmed by Sakai (1985), who demonstrated that *Aeromonas salmonicida* pro-

Table 4. Lysozyme level and index of MPO activity in sick and healthy carps

Experimental group	Type and form of the disease	Age (months)	Lysozyme (μg/ml)	Total protein index
I	erythrodermatitis – acute form	23	2.36 ± 0.32**	2.22 ± 0.29**
II	healthy	23	0.85 ± 0.23	0.97 ± 0.24
III	erythrodermatitis – chronic form	23	0.82 ± 0.23	1.34 ± 0.26**
IV	healthy	23	0.85 ± 0.24	0.97 ± 0.22
V	erythrodermatitis – acute form	28	2.98 ± 0.37**	2.33 ± 0.32**
VI	healthy	28	0.98 ± 0.24	0.97 ± 0.26

**statistically significant increase in the value as compared to the control group

Table 5. Globulin level and level of total protein in sick and healthy carps

Experimental group	Type and form of the disease	Age (months)	Globulins (g/l)	Total protein (g/l)
I	erythrodermatitis – acute course	23	3.64 ± 0.37*	21.63 ± 2. 07*
II	healthy	23	9.94 ± 0.48	30.64 ± 2.11
III	erythrodermatitis – chronic course	23	8.37 ± 0.87*	28.94 ± 3.11*
IV	healthy	23	10.23 ± 0.49	30.72 ± 2.17
V	erythrodermatitis – acute course	28	3.92 ± 0.42*	24.31 ± 3.11*
VI	healthy	28	1.42 ± 0.78	34.12 ± 2.74

*statistically significant decrease in the value as compared to the control group

tease exerts extreme destruction of serum proteins in fishes and by Duswald (quoted according to Evenberg *et al*., 1986), who noted that proteolysis of almost all serum proteins in mammals was associated with the presence of Gram-negative bacteria (and this group includes microbes capable of inducing erythrodermatitis in the fish). Summing up our own results we may state that, irrespectively of the disease form, similar changes take place in globulins and in total protein in the sick carps. It should also be stressed that the phenomenon develops independently of the age and body weight of the fish. It seems that value of the discussed parameters stresses the role and significance of non-specific resistance mechanisms in the fish.

CONCLUSION

Evaluating the obtained and presented above results of studies on carps suffering from acute or chronic form of erythrodermatitis it should be concluded that the age of the sick fishes did not affect the disclosed pattern of resistance in the carps. The observed differences seemed to be associated with chronic or acute course of the disease. The acute or chronic course of the disease affected mainly the metabolic activity of neutrophilic granulocytes (% NTB+ cells, NBT index, amount of formazan) (Table 3) and the serum lysozyme level (Table 4).

REFERENCES

Brzuchowska W. (1966): The use of radioactive isotopes in studies on phagocytosis *in vitro*. Arch. Immunol. Ther. Exp., *14*, 129–146.

Ellis A.E., Hastings T.S., Munro A.L.S. (1981): The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. J. Fish. Dis., *4*, 41–52.

Ellis A.E. (1990): Lysozyme assays. In: Stolen J.S., Fletcher T.C., Anderson D.P., Roberson B.S., Van Muiswinkel W.B. (eds.): Techniques in Fish Immunology. SOS Publications. 101–103.

Evenberg D., de Graaff D., Fleuren W., van Muiswinkel W.B. (1986): Blood changes in carp *(Cyprinus carpio)* induced by ulcerative *Aeromonas salmonicida* infections. Vet. Immunol. Immunopathol., *12*, 321–330.

Kodama H., Hirota Y., Mukamoto M., Baba T., Azuma I. (1993): Activation of rainbow trout *(Oncorhynchus mykiss)* phagocytes by muramyl dipeptide. Dev. Comp. Immunol., *17*, 129–140.

Lamers C.H.J. (1985): The reaction of the immune system of fish to vaccination. [PhD Thesis.] Agricultural University, Wageningen. 256 pp.

Mock A., Peters G. (1990): Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. J. Fish Biol., *37*, 87–885.

Myszkowski L. (1993): Toxic effects of nitrites on protective mechanisms in young developmental forms of the carp (*Cyprinus carpio L*.) and attempts to counteract immunosuppression using Nitrogranulogen preparation (in Polish). [Doctoral Thesis.] Inland Fisheries Institute, Olsztyn.

Park B.H., Fikrig S.M., Smithwick E.M. (1968): Infection and nitroblue-tetrazolium reduction by neutrophils. Lancet, *2*, 532–534.

Prost M. (1991): Fish vaccination – current status of investigations and practical applications (in Polish). Medycyna Wet., *47*, 145–149.

Raman U., Poland R.L. (1975): A new micro-quantitative nitroblue tetrazolium test. Pediatric Res., *9*, 334.

Sakai D.K. (1985): Loss of virulence in a protease-deficient mutant of *Aeromonas salmonicida*. Infect. Immun., *48*, 146–152.

Salwa A. (1980). Attempt of appraising cytochemical and microquantitative technique of NBT reduction test (in Polish). Medycyna Wet., *36*, 737–738.

Siwicki A., Studnicka M. (1987): The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, *Cyprinus carpio* L. J. Fish Biol., *31* (Suppl. A), 57–60.

Siwicki A., Studnicka M., Ryka B. (1985): Phagocytic ability of neutrophils in carp. Bamidgeh, *37*, 123–128.

Siwicki A.K., Anderson D.P., Antychowicz J. (1993): Nonspecific defence mechanisms assay in fish. I. Phagocytic index, adherence and phagocytic ability of neutrophils (NBT test) and myeloperoxidase activity test. In: Siwicki A.K., Anderson D.P., Waluga J. (eds.): Fish Diseases Diagnosis and Prevention Methods (FAO-Project GCP/INT/526/JPN). 95–104.

Stone S.S., Gitter M. (1969): Validity of the sodium sulphite test for detecting immunoglobulins in calf sera. Brit. Vet. J., *125*, 68–73.

Stosik M. (1990): Activity of myeloperoxidase in neutrophilic granulocytes in the course of natural bacterial infection (in Polish). Medycyna Wet., *46*, 440–441.

Stosik M. (1991a): Activity of polymorphonuclear leukocytes during the natural bacterial infection in carps (in Polish). Immunol. Polska, *16*, 131–139.

Stosik M. (1991b): Anatomo-pathological changes and some data on non-specific immunity of carp derived from the fish stock with signs of erythrodermatitis. Medycyna Wet., *47*, 440–442.

Stosik M. (1996). The level of globulins and total protein in sera of healthy carps (*Cyprinus carpio* L*.)*. Medycyna Wet., *52*, 306–308.

Stosik M., Deptuła W. (1990): Mechanisms of specific immunity and of non-specific resistance in fishes (in Polish). Post. Mikrobiol., *29*, 91–102.

Studnicka M., Siwicki A. (1990): The non-specific immunological response in carp (*Cyprinus carpio* L.) during natural infection with *Eimeria subepithelialis*. Bemidgeh, *42*, 18–21.

Svobodova Z., Vykusova B.. (1989): Application of haematological indices in performing test for chronic toxicity (in Czech). In: Celostátní ichtiohaematologická konference, Litomyšl, 22–26.

Sychłowy A., Lukas A. (1978): Evaluation of microquantitative technique of nitroblue reduction test in peripheral blood granulocytes (in Polish). Pol. Tyg. Lek., *33*, 45–47.

Van Muiswinkel W.B. (1992): Fish immunology and fish health. Neth. J. Zool., *42*, 494–499.

Zawistowski S. (1975): Histological Techniques, Histology and Principles of Histopathology (in Polish). PZW, Warszawa. 215 pp.

Received: 99–10–19
Accepted after corrections: 01–01–17

Corresponding Author:

Doc. MVDr. Milan Trávniček, CSc., University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic
Tel. +421 95 633 21 11–15, fax +421 95 632 36 66, e-mail: travnicek@uvm.sk