Original paper

FEED QUANTITY EFFECT ON CARP JUVENILES' PLASMA PROTEIN AND IMMUNOGLOBULIN LEVELS

Relić R.[1], Ardo L.[2], Marković Z.[1], Stanković M.[1], Davidović V.[1], Poleksić V.[1]*

[1] University of Belgrade, Faculty of agriculture, Nemanjina 6, Zemun-Belgrade, Serbia
[2] National Agricultural Research and Innovation Centre, Research Institute for Fisheries, Aquaculture, HAKI, Szarvas, Hungary
*Coresponding author: rrelic@agrif.bg.ac.rs

Abstract
Plasma protein and immunoglobulin (Ig) levels in fish can be influenced by stress reaction, caused by some nutritional factors. This paper shows effects of different feed quantity on total protein and total Ig levels in blood of common carp (*Cyprinus carpio*) juveniles exposed to chronic stress conditions.
The study was carried out in a recirculation aquaculture system (RAS) during 96 days. Carps (initial weight 6.43±0.02 g) were distributed into four groups in three replicate tanks, 40 fish per tank. The groups were formed according to the feed quantity applied: 2, 3, 4 and 5% of commercial extruded mixture in relation to the ichthyomass (i.e. groups I, II, III and IV respectively).
According to the results, the mean values of total plasma proteins and Ig were highest in group IV and the lowest in group II ($p<0.05$). Total protein levels were influenced by feed quantity ($p<0.05$), and sampling time ($p<0.001$). Total Ig levels were influenced by duration of the experiment i.e. sampling time ($p<0.001$). In all groups the mean values of plasma proteins after two month of the experiment was increased ($p<0.01$), and the mean values of Ig in less fed groups I and II ($p<0.05$ and $p<0.01$, respectively). Total plasma proteins and Ig were not significantly affected by water quality parameters only in the group fed 2% of feed (group I). Significant positive correlation between total proteins and Ig was determined in each experimental group ($p<0.01$).
In terms of reducing stress and ensuring welfare in carp juveniles, applied feed amount should match to the length of fish growing in the fish tanks, stocking density and capacity of the system for efficient water purification.

Key words: *common carp, feed quantity, RAS, total immunoglobulins, total proteins*

Introduction

Growing carps in tanks of recirculation aquaculture systems (RAS) implies the absence of natural food and entirely dependence on added feed. Commercial feed must be compliant with the needs of fish, regarding its quality, pellet size, shape and quantity. In deciding the optimal feed quantities for carp juveniles Stanković et al. (2011) considered production and economic indicators (e.g. weight gain, feed conversion ratio and feed price). However, producers' goals must also be directed towards ensuring the welfare of fish.

The level of nutrition is one of the main factors that affect water quality in RAS. Decomposition of uneaten feed and its retention in tanks led to the deterioration of water

quality in the system (Isla Molleda, 2007), which triggers stress response mechanisms in fish (Conte, 2004; Hastein et al., 2005).

Stress reaction involves various physiological changes including alteration in blood composition and immune mechanisms (Wendelaar, 1997). Total plasma protein and immunoglobulin levels were used in the studies of stress response in fish by Cœurdacier and Dutto (1999), Harikrishnan et al. (2003), Ardó et al. (2009), Patriche et al. (2009), Hajibeglou and Sudagar (2010), and Noori (2013). Effects of the feed quantity on these blood parameters in common carp juveniles (*Cyprinus carpio L.*), which are grown in RAS, are not sufficiently examined.

The aim of this paper is to show effects of different feed quantity on total plasma protein and immunoglobulin levels in common carp juveniles kept in tanks over a period of three months.

Material and methods

The study was carried out in recirculation aquaculture system (RAS) of the CEFAH (Center for Fishery and Applied Hydrobiology of the Faculty of Agriculture, University of Belgrade, Serbia). During 96 days 480 specimens of common carp (*Cyprinus carpio*) with the initial weight of 6.43±0.02 g were held in 120-litre tanks with constant water flow of 0.5 l/min. They were distributed in four groups in three replicate tanks, 40 fish per tank. The groups were formed according to the feed quantity applied: 2, 3, 4 and 5% of commercial extruded mixture (38% of proteins and 12% fat, "VZ Subotica", Serbia) in relation to the ichthyomass (i.e. groups I, II, III and IV, respectively). Fish feed was weighed by digital scale (KERN PLS 2100-2, Germany) and distributed by hand, twice a day (08:00–08:30 and 14:00–14:30 hrs).

Water quality in the tanks was checked three times a week (temperature, pH, and dissolved oxygen i.e. DO) by appropriate probe (MULTI 340i/SET, WTW, Germany), and twice a month (unionized ammonia i.e. NH_3-N, and organic substances content based on $KMnO_4$ consumption) by analyzing water samples (Anon., 1985; Čoha, 1990). Water sampling and direct measurements of the water parameters were conveyed in the morning, before feed distribution.

The study had three periods of parameters' examination, and each period was finished at the day of blood sampling. Blood samples were collected from three fish per tank at 32, 64 and 96 day of the experiment. Prior to blood sampling fish were anaesthetized in MS-222 (Sigma-Aldrich®). Methods of sampling blood from caudal vein and blood samples preparation for analyses were described by Roberts (1989), and by Ardó et al. (2009). Blood plasma was isolated by centrifugation (1400G, 15 min), and samples were stored at -20°C before the measurements.

Total protein concentration in the blood was determinated colorimetrically by Fluitest TP (Analyticon® Biotechnologies AG, Germany), as well as total immunoglobulins, with addition of polyethylene glycol (PEG) (Sigma-Aldrich®), as described by Ardó et al. (2008). Both parameters were measured using microplate reader Multiskan Spectrum (Thermo Labsystems; Waltham, MA, US).

As factors affecting blood parameters in this study, the feed quantity and duration of exposure to the same conditions (periods between two consecutive sampling) were considered. Data were analyzed using parametric tests (two-way factorial ANOVA, LSD and Fisher LSD test) at significance level of $p<0.05$. For immunoglobulin levels square root data transformation was applied. Relation between blood and water parameters was

evaluated according to the value of Spearman's rank correlation coefficient. The results were analyzed by STATISTICA 8.0 Software (StatSoft, Inc. 2007), and Microsoft Office EXCEL 2007.

Results and discussion

Based on the results of all blood samplings during the experiment, the highest mean value of total proteins was obtained in group IV, and the lowest in group II ($p<0.05$) (Table 1).

Table 1. *Statistical parameters for total plasma proteins (g/l)*

Groups	Statistical parameters					
	N	Mean*	SE**	Min	Max	C_V (%)**
I	27	38.88 ^{ab}	1.73	19.92	48.18	23.16
II	27	38.12 ^{a}	1.59	22.41	51.62	21.67
III	27	38.94 ^{ab}	1.41	20.26	49.18	18.77
IV	27	42.75 ^{b}	1.15	33.82	56.41	13.96

*a, b – significant at $p<0.05$; the same letter - no significant difference ($p> 0.05$); **SE - standard error; Cv - coefficient of variation

The mean values of total protein were high in all groups, but in group IV was slightly above the upper physiological limit for carp of 40 g/l (according to Svobodová and Vykusová, 1991).

Protein levels could be influenced by the nutritional status (Love, 1980). Patriche et al. (2009) and Noori et al. (2013) found that level of total plasma proteins varies depending on the protein concentration in the feed. In our study, high mean values of the total protein in all groups could be influenced by the high protein level in the feed.

According to results of two-way ANOVA, total protein levels were significantly influenced by feed quantity ($p<0.05$), and period of observation ($p<0.001$), but there was no interaction between these two factors ($p>0.05$). This indicates that blood protein concentration was also influenced by factors other than feed quantities and duration of the experiment. In Table 2 differences of total proteins mean values in each group are shown.

Table 2. *Mean values of the total plasma proteins at the end of the each period (g/l)*

Periods	Groups*			
	I	II	III	IV
first (1^{st} - 32^{nd} day)	28.20 ^{A}	29.17 ^{A}	32.36 ^{A}	37.74 ^{aA}
second (33^{rd} - 64^{th} day)	43.24 ^{B}	42.80 ^{B}	43.58 ^{B}	46.61 ^{B}
third (65^{th} - 96^{th} day)	45.20 ^{B}	42.38 ^{B}	40.90 ^{B}	43.90 ^{b}

*a, b –significant at $p<0.05$; A, B –significant at $p<0.01$; the same letter - no significant difference ($p> 0.05$)

Result shows significant increase in total protein levels in all groups after two months of the experiment i.e. in the second period ($p<0.01$), while these values were not significantly changed in the third period ($p>0.05$).

Increased synthesis of proteins could be associated with tertiary stress response. Stress could be caused by changes in water quality and increased stocking density due to growth of the fish. Plasma composition could be also changed by handling and anaesthetics applied at blood sampling (Ross and Ross, 2008). Smit et al. (1979) found that tricaine methanesulfonate (TMS or MS-222) can induce haemo-concentration in common carp.

This common effect of stress was monitored in several research by measuring plasma protein levels (cited in the paper by Bystriansky et al., 2006).

As in plasma proteins, the highest mean value of total immunoglobulins was in group IV, and the lowest in group II ($p<0.05$). However, in all groups coefficients of variation were greater than in total proteins (Table 3). Largest variations of immunoglobulin levels in group II may result from sporadic occurrence of high concentrations of NH_3-N that was recorded mostly in this group, and which could adversely affect the activity of the carps' immune system (Wlaslow et al., 1990). Cœurdacier and Dutto (1999) found in their study significantly lower level of total immunoglobulins in fish exposed to high level of ammonia compared with fish from the control group.

Table 3. *Statistical parameters for total immunoglobulins (g/l)*

Groups	Statistical parameters					
	N	Mean*	SE**	Min	Max	C_V (%)**
I	27	18.01 ^{ab}	0.90	4.83	25.91	25.89
II	27	15.85 ^{a}	1.18	3.88	25.85	38.83
III	27	17.34 ^{ab}	1.13	1.99	31.33	33.93
IV	27	19.17 ^{b}	0.99	4.64	29.74	26.88

*a, b –significant at $p<0.05$; the same letter - no significant difference ($p> 0.05$); **SE - standard error; Cv - coefficient of variation

Feed quantity (itself and in interaction with sampling) had no significant influence on total immunoglobulin levels ($p>0.05$), unlike to the period of observation ($p<0.001$). Similar to plasma proteins, absence of the interaction between feed quantity and period of observation could indicate the influence of other factors, including quality of the water in tanks, increased stocking density and the presence of bacteria in the water.
Primary and secondary stress responses are short-term effects of acute, short-lived challenges. When these responses are prolonged or repeated and fish has no way to avoid or escape the challenge, a series of tertiary effects become apparent, including changes in immune function (cited by Broom DM in EFSA, 2008). Influence of the observation periods was manifested in all groups except in group III (Table 4).

Table4. *Mean values of the total plasma immunoglobulins at the end of the each period (g/l)*

Periods	Groups*			
	I	II	III	IV
first (1st - 32nd day)	14.83 ^{a}	11.27 ^{a}	16.84 ^{a}	17.00 ^{a}
second (33rd - 64th day)	19.45 ^{ab}	19.07 ^{B}	19.38 ^{a}	22.89 ^{b}
third (65th - 96th day)	19.76 ^{b}	17.20 ^{B}	15.81 ^{a}	17.61 ^{ab}

*a, b – significant at $p<0.05$; A, B – significant at $p<0.01$; the same letter - no significant difference ($p> 0.05$)

Results from the table indicate that in the less fed groups I and II a significant increase in the amount of immunoglobulin occurred ($p<0.05$ and $p<0.01$, respectively). On the other hand, although decrease of the values in groups II, III and IV in the third period was not significant, it could be result of chronic exposure to stressors (Dobšíková et al., 2009; Magnadottir et al., 2010), considering the suppressive effects on the immune response of fish.

According to previously published data from the same experiment, increased feed quantity was affected fish average body mass (Stanković et al., 2009), and the water quality was gradually deteriorated (Relić, 2011; Relić et al., 2011) especially in groups III and IV in

the third period. Table 5 shows that total protein and immunoglobulin concentration was significantly affected by at least one water quality parameter in all experimental groups, except for group I.

Table 5. *Significant correlation between water and blood parameters at group level*

Parameters	Groups**							
	I		II		III		IV	
	ρ*	p-level	ρ	p-level	ρ*	p-level	ρ	p-level
temp & prot	-0.163	0.418	*-0.409*	**0.034**	-0.214	0.285	-0.223	0.263
temp & Ig	-0.233	0.241	*-0.430*	**0.025**	-0.296	0.134	-0.072	0.723
DO & prot	0.345	0.078	*0.392*	**0.043**	*0.425*	**0.027**	0.117	0.562
NH_3-N & prot	-0.486	0.329	*0.886*	**0.019**	-0.486	0.329	0.543	0.266
organic sub. & Ig	0.754	0.084	0.319	0.538	0.086	0.872	*-0.886*	**0.019**
prot & Ig	*0.600*	**0.001**	*0.857*	**<0.001**	*0.604*	**0.001**	*0.572*	**0.002**

*ρ – Spearman's rank correlation coefficient**;** **bolded values are statistically significant

Total proteins were in the significant negative correlation to the water temperature in group II, and positively correlated to DO (groups II and III) and NH_3-N concentration (group II). Total immunoglobulin values were significantly and negatively correlated to the water temperature (group II) and organic matter content (group II). Correlation between total proteins and immunoglobulins was significant and positive in the each experimental group ($p<0.01$), and strongest in group II.

Conclusions

According to the results from this study, feed quantity has showed influence on total protein levels, while period of observation i.e. time of sampling had influence on both parameters, total protein and immunoglobulin levels.

Rearing conditions (limited space, increase of feed quantity with increasing of the ichtyomass, increasing of stocking density, and consequently deterioration of the water quality over the time) have created a state of chronic stress.

In terms of reducing stress and ensuring welfare in carp juveniles, applied feed amount should match to the length of fish growing in the fish tanks, stocking density and capacity of the system for efficient water purification.

Acknowledgements

This paper is supported by project TR 31075 of the Ministry of Education, Science and Technological Development, Republic of Serbia.

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