LIVER HISTOLOGY AND VARIATION OF HEPATOCYTES NUCLEAR AREA OF RAINBOW TROUT (*Oncorhynchus mykiss*) REARED IN CAGES

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HISTOLOGIJA JETRE I VARIRANJE POVRŠINE JEDARA HEPATOCITA PASTRMKE GAJENE U KAVEZNOM SISTEMU

Abstrakt

U hidroakumulacionom jezeru ”Bočac” gajena je kalifornijska pastrmka (Oncorhynchus mykiss, Walbaum, 1792) u dva odvojena eksperimenta u trajanju od po 90 dana – jedan u periodu jesen – zima, a drugi u periodu proleće – leto. Pastrmke su hranjene sa šest različitih komercijalnih hraniva i ispitivan je njihov uticaj na histološku gradju jetre riba. U eksperimentu je preovladavala normalna histološka građa jetre, a malobrojne histopatološke promene koje su uočene se mogu pripisati periodu godine i sastavu hrane. Kvantifikacija rezultata je pokazala da se sa rastom temperature vode i količine hrane kojom su ribe hranjene, prosečna površina jedara hepatocita povećava, dok se sa opadanjem temperature i količine hrane prosečna površina jedara hepatocita povećava, nezavisno od tipa hrane koja je korišćena.

***Ključne reči**: kalifornijska pastrmka, kavezni system, histologija jetre, temperature, hrana*

INTRODUCTION

Fish from aquaculture often have alterations in vital organ morphology. As function of food availability, season, species, and/or environmental conditions modifications in histological structure of digestive organs occur (S t r u s s m a n n and T a k a s h i m a, 1990; T a k a s h i m a and H i b i a, 1995; C a r r i q u i r i b o r d e et al. 2007). Liver plays a key role in metabolism of nutrients absorbed in the digestive tract (O l s v i k et al. 2007). Morphological effects on the liver depend on fish species. A number of studies

were carried out on nucleoli of eurythermal species showing increase of liver metabolism alterations with sudden change of temperature (A l v a r e z et al. 2006; C a r r i q u i r i b o r d e et al. 2007; I t o i et al. 2003, S a r m i e n t o et al. 2000). Eurythermal fish have evolved compensatory responses to cope for the naturally occurring seasonal changes which affect their habitat. Although temperature and photoperiod are the main physical factors that distinguishes summer from winter, food availability, among others also influence the adaptive regulation of fish (A l v a r e z et al. 2004). Studies monitoring nuclear area of hepatocytes in rainbow trout are mainly stereological studies of normal liver (R o c h a et al. 1997), or studies concerning breeding cycle: variation mainly attributed to sex differences (R o c h a et al. 2009), starvation experiments (P o w e r et al. 2000), effects of different feed types (P o l e k s i c et al. 2006; O s t a s z e w s k a et al. 2005), and ecotoxicological studies (H o f e r et al. 1999).

In the previous 60 days experiment with different commercial feed used for trout reared in cages, a statistically significant decrease of hepatocytes nuclear area was found in the period spring - summer (P o l e k s i c et al. 2006). The present study was carried out in order to investigate the effect of temperature and food quantity on liver histology and hepatocytes nuclear area of trout cultured in cage system.

MATERIAL AND METHODS

Experiments were performed in two 90-days periods. Rainbow trout yearlings had an individual mass from 93.97 to 99.43 grams (Tab. 1). The first cycle was carried out in the period autumn – winter (A/W) (19/10/2005 – 24/01/2006), and a second in the period spring – summer (S/S) (02/04/2006 – 01/07/2006). Experimental design was the following: 6 (six) identical cages of the cage system "Tropik" in the hydro accumulation Bočac in Bosnia and Herzegovina were used. Cages dimensions were 5 x 5 x 7 m, useful production volume of 162.5 m^3. Cages were marked as 1, 2, 3, 4, 5, and 6, as were six different commercial compound feeds. Each cage was stocked with 400 kg rainbow trout. Fish were fed six different commercial feed types; their composition is given in Tab. 2.

Table. 1. Average individual weight of rainbow trout per treatment at the beginning of the first and second period.

Cycle	1	2	3	4	5	6
I	96.28	94.75	97.95	94.29	94.56	93.97
II	94.08	97.32	95.04	99.43	96.33	94.39

Water quality was monitored during the experiment and was within the first class of quality. Water temperature and oxygen concentration were measured daily at 1, 2, and 3 m depth (1, 2 i 3 m) using the Oxi 330i/SET 2B20-0011 WTW, Germany.

Table. 2. Feed composition used in the experiment.

Food type	**1**	**2**	**3**	**4**	**5**	**6**
Crude proteins, %	44,0	48,0	42,0	42,0	44,0	42,0
Crude fat, %	14,0	26,0	22,0	23,0	26,0	18,0
Crude fiber, %	5,0	1,0	3,3	1,8	1,3	1,7
Ash, %	9,0	8,5	10,0	8,0	10,0	8,8
Phosphorus, %	1,2	0,9	1,3	1,1	1,5	1,2
Vitamine A (IU/kg)	6000	15000	6000	15000	6000	15000
Vitamine E(mg/kg)	200	200	200	200	200	200
Copper (mg/kg)	3,0	5,0	3,0	5,0	3,0	5,0
Bruto energy(MJ)	20,4	23,8	21,8	22,3	23,2	21,0
Digestible energy (MJ)	17,7	21,9	19,3	20,3	20,9	19,1
Metabolic energy (MJ)	15,7	19,6	17,4	18,3	18,9	17,2
Nitrogen-Free Extract, %	21,0	17,0	15,0	17,2	13,0	21,5

Samples for histological analysis were taken prior to experiment beginning and after 90 days. Three fish per cage were sacrificed, and samples of the liver fixed in 4 percent formaldehyde and embedded in paraffin. For histological analysis slides were prepared using a standard histological technique with hematoxiline/eosine staining of 5 μm sections. Slides were examined under the Leica DM LS light microscope, with a DC 300 camera. Morphometric parameters were measured using a Leica IM 1000 program. Average nuclear area of 30 hepatocytes per each liver section was determined. At least 5 sections per liver sample were analyzed.

Statistical analysis was done using a Microsoft Office Excel 2003 program: Statistical Analysis Tools (ANOVA: Single Factor I t-Test: Two-Sample Assuming Equal Variances).

RESULTS AND DISCUSSION

Temperature varied from 4.8 °C (in the last 15 days) to 11.96 °C (in the first 15 days of the experiment) during the autumn – winter (A/W) period (Fig.1), while in the spring - summer (S/S) period variations were from 8.61 (in the first 15 days) till 19.37 °C (in the last 15 days) (Fig.2). In accordance with seasonal changes average values of temperature in the first period (A/W) have shown gradual decrease, and in the second period (S/S) gradual increase.

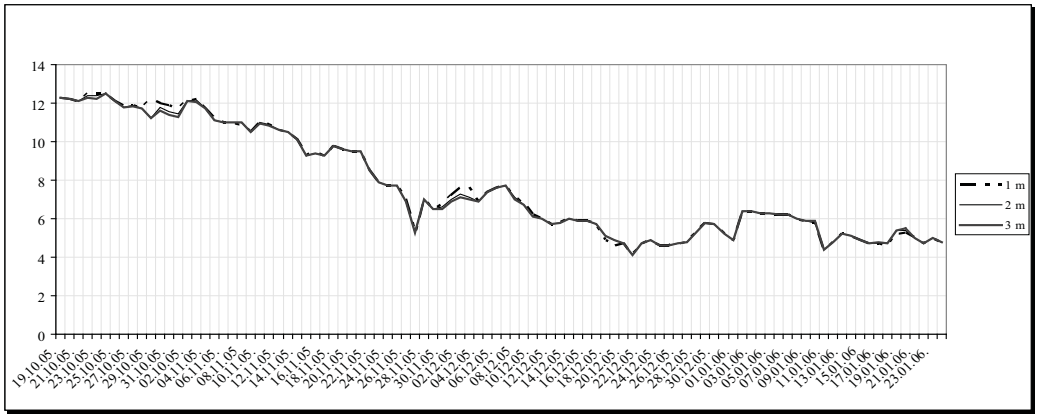

Figure 1. Temperature variation during the autumn – winter period (A/W).

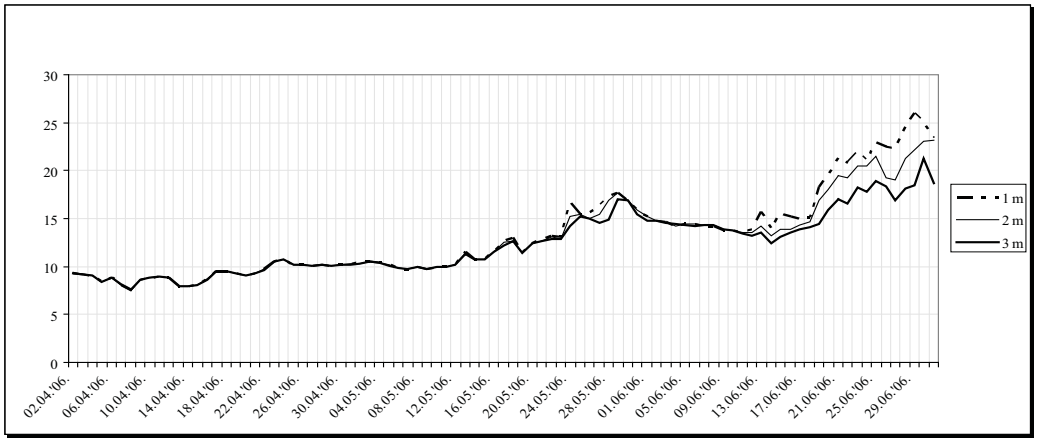

Figure 2. Temperature variation during the spring – summer period (S/S).

Liver histology

Normal histological structure of the liver was dominant on most of the samples examined. Changes such as hepatocytes vacuolization, occasionally fatty changes, congestion of sinusoids and larger blood vessels were found in both A/W and S/S experiments (Fig. 3 and 4). Vacuolated hepatocytes and other signs of fatty liver degeneration were found on livers of fish fed feed 2 and 5 in A/W period. Liver of fish fed feed type 4 had, in addition to the mentioned symptoms, signs of fibrosis. Similar alterations were noticed in the S/S experiment on livers of trout fed feed 2 and 5 when fish consumed greater quantity of food. In addition to these changes livers of trout fed feed 2 and 5 had some picnotic nuclei of hepatocytes. Picnotic nuclei were found on livers of fish fed feed number 1 and 6 as well (S a v i c et al. 2008).

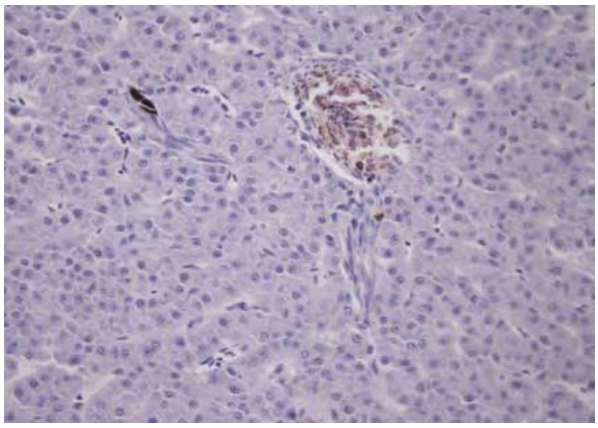

Figure 3. Congestion of blood vessels

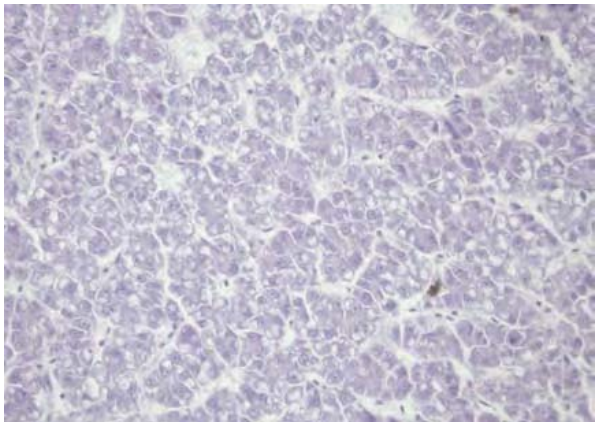

Figure 4. Hepatocytes vacuolization

In conclusion, in both A/W and S/S part of the season signs of fatty liver degeneration appeared in trout fed commercial feed with the highest lipid (26 %) and protein level (48 % and 44 % for feed 2 and 5, respectively). High lipid content in the compound feed could cause fatty degeneration of the liver (C a b a l l e r o et al. 2003; D u et al. 2008). Nuclear picnosis is an effect of fat accumulation in liver cells and their degeneration (T a k a s h i m a and H i b i y a, 1995). Livers of trout fed feed type 5 in both periods (A/W and S/S) had the best growth rate although with liver fatty degeneration. Best food conversion ratio and best Fulton's condition coefficient were observed, again, in cages with trout fed feed 5 and 2 that had a highest lipid level (S a v i c et al. 2008). A z e v e d o et al. (2004) found that food consumption increases with decrease of protein content and increase of lipids.

Hepatocytes nuclear area measurement

During the first period (A/W) as shown on Tab.3 in all cages hepatocyte nuclear area had an increasing trend from the beginning of the experiment. Statistically significant ($\alpha = 0.05$ and $\alpha = 0.01$) increase of the average hepatocytes nuclear area was determined

using t-test, with maximum increase in the cage 6, where the increase of average hepatocytes nuclear area of 12.25 μm^2 was found at the end of the experiment compared to its beginning. Least increase of average hepatocytes nuclear area of 6.11 μm^2 (end of experiment compared to the beginning) was found in cage 5.

Table 3. Average hepatocytes nuclear area values (μm^2), average differences (μm^2), coefficient of variation and t - test (Two-Sample Assuming Equal Variances) between the start and end of the autumn – winter period (A/W)

Cage	$\bar{x}_{start}$	$\bar{x}_{end}$	$\bar{x}_{start} - \bar{x}_{end}$	V_{start}	V_{end}	$t_{calc.}$	t_{tab}	
							0.05	0.01
1	*20.308*	*32.283*	-11.98**	13.434	17.838	-27.42**	1.96	2.59
2	*22.689*	*31.449*	-8.76**	11.737	16.906	-21.56**		
3	*22.057*	*30.357*	-8.30**	12.536	23.986	-15.39**		
4	*21.217*	*28.856*	-7.64**	11.995	16.664	-20.62**		
5	*22.955*	*29.067*	-6.11**	12.451	13.414	-19.02**		
6	*18.627*	*30.876*	-12.25**	14.445	14.229	-35.26**		

Average hepatocyte nuclear area at the end of the second experiment, as shown on Tab.4, decreased in all cages compared with values at experiment start. The slightest difference of average hepatocytes nuclear area of 0.75 μm^2 was found in cage number 5, while the biggest difference was 6.67 μm^2 in cage number 1. In the second experiment a statistically significant decreasing trend in hepatocytes nuclear area, compared to the control was detected ($\alpha = 0.05$ and $\alpha = 0.01$).

Table 4. Average hepatocytes nuclear area values (μm^2), average differences (μm^2), coefficient of variation and t - test (Two-Sample Assuming Equal Variances) between the start and end of the spring – summer period (S/S)

Cage	$\bar{x}_{start}$	$\bar{x}_{end}$	$\bar{x}_{start} - \bar{x}_{end}$	V_{start}	V_{end}	$t_{calc.}$	t_{tab}	
							0.05	0.01
1	*26.172*	*19.499*	6.67**	13.39	9.89	27.35**	1.96	2.59
2	*25.433*	*19.636*	5.80**	18.10	11.39	18.75**		
3	*23.975*	*18.294*	5.68**	16.19	12.80	20.39**		
4	*24.067*	*19.154*	4.91**	14.18	11.94	19.28**		
5	*20.298*	*19.552*	0.75*	20.25	12.80	2.52*		
6	*24.637*	*20.189*	4.45**	15.47	14.59	14.68**		

Using F-test (two factorial experiment 2 x 6; two experiments x 6 feed types; hepatocytes nuclear area) a statistically significant ($\alpha = 0.05$ and $\alpha = 0.01$) difference in hepatocytes nuclear area was determined (Tab.5). The results have shown that average hepatocytes nuclear area is highly affected by the season i.e. water temperature and daily feed quantity. With increase of temperature and feed quantity hepatocytes nuclear area decreased and it increases with the decrease of temperature and feed quantity.

Table 5. Effect of the season and feeding on hepatocytes nuclear area at the end of first and second experiment and factor interaction - two factorial model (ANOVA).

Type of variation	*F* calc.	*F* tab.	
		0.05	*0.01*
Factor A (experiment)	6.438.86**	3.84	6.64
Factor B (hepatocytes nuclear area)	22.79**	2.22	3.02
Interaction	15.54**		

Similar results were obtained in the experiment that lasted 60 days from 17/05/2005 to 17/07/2005 (S/S). This study was carried out in the same lake of the hydro accumulation Bočac using the same commercial feeds (P o l e k s i c et al. 2006*)*. It was a 60 days experiment, but average values of hepatocytes nuclear area decreased as in the S/S period of the 90 days experiment year later.

In rainbow trout fatty acid composition and temperature are important for the metabolism of lipids (T o c h e r et al. 2004). The results obtained in the present and the previous study (P o l e k s i c et al. 2006) are pointing out changes in hepatocytes metabolism along the season. The reason for the nuclear area increase could be attributed to increased protein synthesis, and therefore enlarged hepatocytes nuclei in the period of exhaustion of carbohydrates and lipids as energy sources in A/W period. In the S/S period when temperature raise and feed quantity increase, protein synthesis and therefore nuclear area diminish. It is known that the nuclear area of hepatocytes is directly related to DNA content (N u n e z et al. 2000). Moreover, the energy source during decreased feeding in fish varies among species: some using mainly glycogen while others use lipids or proteins. Energy mobilization during food restriction may be affected by other factors, including ambient temperature and may provoke alterations in tissue structure (S o u z a et al. 2001).

The ability to adapt to the changes in resource availability by mobilization of energy -providing substrates occurs in order to support the body’s requirements, although the use of lipids, protein and glycogen leads to cellular modifications in fish tissues (S o u z a et al. 2001, R i o s et al. 2007).

CONCLUSION

The results of the study of liver histology have shown that in trout fed six different commercial diets mainly normal histological structure prevailed with some alterations that could be attributed to the season and feed composition.

In order to adapt to environmental conditions a season-dependent change in average hepatocytes nuclear area occurs in the rainbow trout.

Effect of temperature and feed quantity was negatively correlated to hepatocytes nuclear area: average values of the nuclear area of hepatocytes decreased with the increase of temperature and feed quantity, and it increased when temperature and feed quantity decreased, regardless of feed composition.

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REFERENCES

Alvarez, M., Molina, A., Quezada, C., Pinto, R., Krauskopf, M. and Vera M. I. (2004). Eurythermal fish acclimatization and nucleolar function: a review. Journal of Thermal Biology 29, 663-667

Alvarez M., Quezada C., Molina A., Krauskopf M., Vera M. I. and Thiry M. (2006). Ultrastructural changes of the carp (Cyprinus carpio) hepatocyte nucleolus during seasonal acclimatization. Biology of the Cell 98, 457-463

Azevedo, P. A., Leesona, S., Cho, C. Y. and Bureaua, D. P. (2004). Growth, nitrogen and energy utilization of juveniles from four salmonid species: diet, species and size effects. Aquaculture 234, 393–414.

Caballero, M. J., Izquierdo, M. S., Kjorsvik, E., Montero, D., Soccoro, J., Fernandez, A. J. and Rosenlund, G. (2003). Morphological aspects of intestinal cells from githead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225, 325-340.

Carriquiriborde, P., De Luca, J. C., Dulout, F. N. and Ronco, A. E. (2007). Nucleolar variation in response to nutritional condition in juvenile pejerrey Odontesthes bonariensis (Valenciennes). Journal of Fish Biology 70, 947–958

Du, Z. Y., Clouet, P. , Huang, L. M. , Degrace, P., Zheng, W. H. , He, J. G., Tian, L. X. and Liu, Y. J. (2008). Utilization of different dietary lipid sources at high level in herbivorous grass carp (*Ctenopharyngodon idella*): mechanism related to hepatic fatty acid oxidation. Aquaculture Nutrition 14; 77–92.

Hofer R., Kock G. and Braunbeck T. (1997). Nuclear alterations in hepatocytes of Arctic char Salvelinus alpinus from acidic high alpine lakes. Diseases of Aquatic Organisms 28, 139-150

Itoi S., Kinoshita S., Kikuchi K. and Watabe S. (2003). Changes of carp FoF1-ATPase in association with temperature acclimation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 284, R153-R163

Nunez, F.,Chipchase, M. D., Clarke, A. R., & Melton, D.W. (2000): Nucleotide excision repair gene (ERCC1) deficiency causes G2 arrest in hepatocytes and a reduction in liver binucleation: the role of p53 and p21. *FASEB J.* 14, 1073–1082 (2000)

Olsvik, P. A., Lie, K. K., Sæle, Ø.and Sanden, M. (2007). Spatial transcription of CYP1A in fish liver. *BMC Physiology* 2007, **7**:12.

Ostaszewska, T., Dabrowski, K., Czuminska, K., Olech, W. and Olejniczak, M. (2005). Rearing of pike-perch larvae using formulated diets - first success with starter feeds. Aquaculture Research 36, 1167-1176

Power, D. M., Melo, J. and Santos, *C. R. A.* (2000). The effect of food deprivation and refeeding on the liver, thyroid hormones and transthyretin in sea bream. Journal of Fish Biology 56, 374–387.

Poleksić, V., Savić, N., Rašković, B., Marković, Z. (2006). Effects of different feed types on histology of intestine and liver of the cage cultured trout. Biotechnology in animal husbandry, Vol 22, Special issue 359-372. *(in Serbian)*

Rios, F. S., Donatti, L., Fernandes, M. N., Kalinin, A. L. and Rantin, F. T. (2007). Liver histopathology and accumulation of melano-macrophage centres in *Hoplias malabaricus* after long-term food deprivation and re-feeding. Journal of Fish Biology 71, 1393–1406

Rocha, E., Monteiro, R. A. F. and Pereira, C. A. (1997). Liver of the brown trout, *Salmo trutta* (Teleostei, Salmonidae): A stereological study at light and electron microscopic levels. Anatomical Record 247, 317-328

Rocha, E., Rocha, M. J., Galante, M. H., Silva, M. W. and Monteiro, R. A. F. (2009). The hepatocytes of the brown trout (*Salmo trutta m. fario*): a stereological study of their number and size during the breeding cycle. Ichthyological Research 56, 43-54

Sarmiento, J., Leal, S., Quezada, C., Kausel, G., Figueroa, J., Vera, M. I. and Krauskopf, M. (2000). Environmental acclimatization of the carp modulates the transcription of beta-actin. Journal of Cellular Biochemistry 80, 223-228

Savić, N., Marković, Z., Rašković, B., Poleksic, V. (2008). Effect of different feed composition on productive results of trout (*Oncorhynchus mykiss*, Walbaum,1792) reared in cages. Biotechnology in Animal Husbandry 24, Special issue, p.285-292. *(in Serbian)*

Souza, V. L., Lunardi, L. O., Vasques, L. H., Casaletti, L., Nakaghi, L. S. O. and Urbinati, E. C. (2001). Morphometric alterations in hepatocytes and ultrastructural distribution of liver glycogen in pacu (*Piaractus mesopotamicus* Holmberg, 1887) during food restriction and refeeding. *Braz. J. morphol. Sci 18(1), 15-20.*

Strussmann, C. A. and F. Takashima (1990). Hepatocyte nuclear size and nutritional condition of larval pejerrey, *Odontesthes bonariensis* (Cuvier et Valenciennes). J. Fish Biol. 36, 59-65.

Takashima, F. and Hibia, T. (1995). An atlas of fish histology. Normal and pathological features. Kodansha. Gustav Fisher Verlag p. 195.

Tocher, D. R., Fonseca-Madrigal, J., Dick, J. R., Ng, W., Bell, J. G., Campbell, P. J. (2004). Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part B 137, 49–63.