PERIODICUM BIOLOGORUM
VOL. 108, No 2, 127–131, 2006

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Plasma metabolites and enzymes of bluefin tuna, *Thunnus thynnus* and liver histology

NATALIJA TOPIĆ POPOVIĆ
IVANČICA STRUNJAK-PEROVIĆ
ROZELINDRA ČOŽ-RAKOVAC
MATO HACMANJEK

Ruđer Bošković Institute,
Division of Material Chemistry
Ichthyopathology Group – Biological Materials
Bijenička 54, 10000 Zagreb, Croatia

Correspondence:
Natalija Topić Popović,
Ruđer Bošković Institute,
Division of Material Chemistry,
Ichthyopathology Group – Biological Materials
Bijenička 54, 10000 Zagreb, Croatia
E-mail: ntopic@irb.hr

Key words: *Thunnus thynnus*, blood biochemistry, liver histology

Abstract

Background and Purpose: *Virtually no comprehensive study has been published on tuna plasma chemistry parameters and their correlation with tissue histological status. This study will be useful for establishing plasma biochemistry reference values for bluefin tuna.*

Materials and Methods: *Heparinized blood samples were collected from bluefin tuna. Plasma biochemical analyses were performed using standard techniques. Organ tissues were processed histologically and stained with hematoxylin/eosin. Mean, median, standard deviation, standard error of mean, 25th percentile, 75th percentile and range difference of minimum and maximum values were calculated for each variable measured*

Results: *Obtained values represent preliminary results. All blood biochemistry parameters, except ALT, passed Kolmogorov-Smirnov normality test. In a number of hepatocytes, different degrees of fatty changes were visible.*

Conclusions: *Normal ranges of plasma activity vary between species and normal values must be produced from a representative population of each species. This study will be followed by other studies on blood chemistry values of bluefin tuna in the Adriatic Sea involving more individuals from several locations, that could provide a basis for reference levels.*

Received March 1, 2006.

INTRODUCTION

Atlantic bluefin tuna (*Thunnus thynnus*) farming in the Adriatic Sea (Croatia) implies caging of wild tuna for fattening, mainly for Japanese market. Tuna farming involves capture of wild stock and aquaculture practice where fish are bred and reared in captivity. According to the Croatian Chamber of Economy, Croatian tuna farming in 2002 reached 4000 tons, and exceeded sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labarax*) production of 2700 tons. With secured export, tuna has become one of the most important export products of agriculture and food industry in Croatia. The growth of tuna production is limited within national catching quota, which all ICCAT (The International Commission for the Conservation of Atlantic Tunas) members must respect. Spain and Croatia are the leading countries in this new tuna farming model. During the last 3–5 years there has been a very important development of tuna farms in the Mediterranean, currently amounting to about 20 farms (*1*). Due to increased farming, there is a growing interest in tuna diseases and clinical data, which are not yet available.

The evaluation of blood chemistry parameters in animals is an important tool in clinical practice, which can provide essential information on the physiological status of the animal. In fish, however, the predictive value is compromised by the lack of normal reliable databases and available reference laboratories to properly analyze these samples (*2*). Also, there is a lack of databases for tuna (*3*). Some baseline hematologic parameters are available for albacore (*Thunnus alalunga*), skipjack (*Katsuwonus pleamis*) and yellowfin (*Thunnus albacares*) tuna (*4, 5*). In Croatia, most of blood chemistry analyses of sea-farmed fish have been conducted on sea bass and salmonid species (*6, 7*). Experimental approach to reveal the link between disease state, histopathology and blood chemistry is still urgently needed for many teleost species (*8*).

This study is part of a project on investigating aquacultured wild-caught seedstock in the mid-Adriatic Sea through selected hematological parameters and histological findings. Although tuna farming is becoming ever more important, few studies have been undertaken to investigate the effect of captivity and farming on their condition, mainly due to reluctance of investors to submit valuable tuna for scientific investigation.

MATERIALS AND METHODS

Six Atlantic bluefin tuna *Thunnus thynnus* (average weight 100 kg, average length 125 cm) were randomly sampled from a Croatian floating-cage farm located in the mid-Adriatic Sea. Cages were 50 m in diameter and 32 m or 38 m in depth. Every cage contained approximately 1000 specimens. Wild tuna were originally captured by seine net in April 2003 in the waters off Malta, towed, and transferred to the farm in the Adriatic Sea. They were towed for 40 days before reaching the farming site and not fed while towed. A week after reaching the final site, the feeding commenced. Tuna were fed sardine (*Sardina pilchardus*) and defrosted herring (*Clupea harengus*) according to their size. The age of specimens examined in this study was *c.* 5 years and 6 months as estimated by standard length and body weight. Fish were not fed any medications. In November 2003, fish for this study were rapidly sacrificed and immediately bled. Long syringes were used for blood withdrawal from the caudal artery and vein. Blood was collected in tubes coated with anticoagulant lithium heparin, centrifuged at 12000 x g for 90 seconds and resultant plasma was frozen at –20 °C for storage until analysis. Total proteins (TP), albumin (ALB), globulin (GLOB), plasma triglyceride (TRIG), cholesterol (CHOL), glucose (GLU), urea (URE), NH_3, alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT) levels were determined using a biochemical analyzer (VETTEST 8008, USA). The analyzer, originally designed for clinical tests on mammalian serum or plasma, was adapted to measure variables for fish plasma. Reactions needed for a single test occurred within a multilayered dry test slide identifying test that was run. A sample was drawn into the automatic pipette tip and 10 µl dispensed onto each slide. As the sample filtered through the layers, biochemical reactions took place within the film producing progressive color changes. The system uses three reflectometers operating at 6 wavelengths to perform both end point and rate measurements. If results of a particular test were »above the range of the analyzer«, the plasma was diluted with distilled water and reanalyzed.

A complete necropsy was prerformed on each fish, external and gross abnormalities were recorded. Tissues of liver, spleen, kidney, brain and gonads were fixed in 4% neutral buffered formalin, dehydrated through a graded ethanol-xylene series and embedded in paraplast. Sagittal and transverse sections, 3–5 µm thick, were stained with hematoxylin/eosin (H&E).

Statistical analyses were performed using Sigma Statistical Software, Version 1.0. Mean, median, standard deviation, standard error of mean, 25th percentile, 75th percentile and range difference of minimum and maximum values were calculated for each measured variable.

RESULTS

The fish under examination did not manifest any external trauma, scars or injuries, which can be attributed to applying good husbandry principles such as not towing cages at an excessive speed during fish transfer and keeping fish at reduced stocking densities.

Individual values for every blood parameter of each fish in assay are presented in Table 1. All blood biochemistry parameters, except ALT, passed Kolmogorov-Smirnov normality test, indicating that the data matched the pattern expected if representing a population with normal distribution. Pearson Product Moment Correlation revealed that GLU values tended to increase or decrease together with urea, NH_3 and LDH variables, while it demonstrated no significant relationship of GLU with other variables. Also, it computed for CHOL and CK that one variable tended to decrease while the other increased. One particular specimen (No. 6) exhibited significantly higher levels of urea, GLU, NH_3, LDH and AST when compared to other fish. Table 2 shows the mean, median, standard deviation (SD), standard error of mean (SE), 25th percentile, 75th percentile and range difference (Rng. Diff.) of minimum and maximum values for each blood chemistry parameter. These values were determined because normal distribution was observed for some of the parameters whereas others were skewed. The parameters of cardiac, hepatic, lipid and renal profiles of tuna in assay were not significantly elevated or reduced when mutually compared.

Histologically, lobular architecture of liver was not visible. Hepatocytes were polygonal with spherical nuclei often located eccentrically, with granular and acidophilic cytoplasm. In a number of hepatocytes, different degrees of fatty lesions were visible (Figure 1). Other examined tissues demonstrated no aberrations from normal histological status.

TABLE 1

Blood parameters of six individual bluefin tuna randomly sampled from an Adriatic floating-cage farm.

	Individual specimens					
Blood parameter	1	2	3	4	5	6
TP ($g\ l^{-1}$)	66	67	77	85	84	70
ALB ($g\ l^{-1}$)	21	24	23	28	26	24
GLOB ($g\ l^{-1}$)	44	44	54	58	57	46
GLU ($mmol\ l^{-1}$)	9.08	7.03	11.66	9.75	10.12	15.86
CHOL ($mmol\ l^{-1}$)	3.64	2.31	3.83	3.37	2.74	3.27
TRIG ($mmol\ l^{-1}$)	1.83	1.99	2.32	2.1	3.16	2.24
URE ($mmol\ l^{-1}$)	0.09	0.00	0.22	0.07	0.22	0.36
NH_3 ($\mu mol\ l^{-1}$)	390	440	701	583	609	950
ALKP ($U\ l^{-1}$)	34	37	58	47	14	54
LDH ($U\ l^{-1}$)	500	>11,200	1,307	2,680	>11,200	2,800
CK ($U\ l^{-1}$)	265	2,036	457	1,154	2,860	974
GGT ($U\ l^{-1}$)	0	0	0	0	0	0
AST ($U\ l^{-1}$)	0	351	38	44	209	646
ALT ($U\ l^{-1}$)	10	24	<10	<10	<10	22

TABLE 2

Descriptive statistics of enzyme activities and metabolite concentrations in plasma of six bluefin tuna.

Blood parameter	Mean	Median	SD	SE	Rng.Diff.	25%	75%
TP ($g\ l^{-1}$)	74.58	73.50	8.42	3.44	19.00	67.00	84.00
ALB ($g\ l^{-1}$)	24.33	24.00	2.42	0.99	7.00	23.00	26.00
GLOB ($g\ l^{-1}$)	50.50	50.00	6.57	2.68	14.00	44.00	57.00
GLU ($mmol\ l^{-1}$)	10.58	9.93	2.99	1.22	8.83	9.08	63.50
CHOL ($mmol\ l^{-1}$)	3.19	3.32	0.57	0.23	1.52	2.74	3.64
TRIG ($mmol\ l^{-1}$)	2.27	2.17	0.47	0.19	1.33	1.99	2.32
UREA ($mmol\ l^{-1}$)	0.16	0.16	0.13	0.05	0.36	0.07	0.22
NH_3 ($\mu mol\ l^{-1}$)	612.17	596.00	200.94	82.03	560.00	440.00	701.00
ALKP ($U\ l^{-1}$)	40.67	42.00	16.05	6.55	44.00	34.00	54.00
LDH ($U\ l^{-1}$)	4,947.83	2,740.00	4,918.83	2,008.10	10,700.00	1,307.00	11,200.00
CK ($U\ l^{-1}$)	1,291.00	1,064.00	988.36	403.50	2,595.00	457.00	2,036.00
AST ($U\ l^{-1}$)	214.67	126.50	249.43	101.83	646.00	38.00	351.00
ALT ($U\ l^{-1}$)	14.33	10.00	6.74	2.75	14.00	10.00	22.00

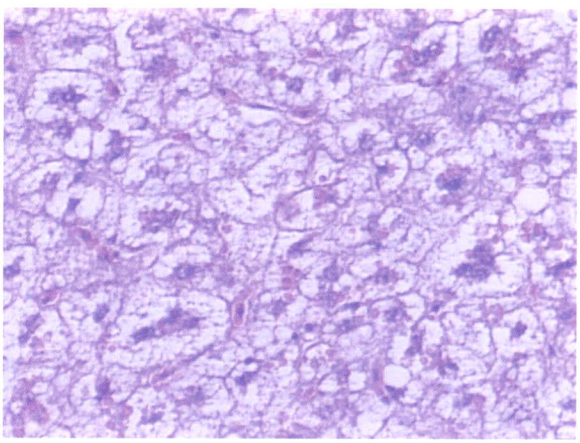

Figure 1. Thunnus thynnus *liver. H&E x100. Fatty changes within hepatocytes and degenerative cells.*

DISCUSSION

Plasma biochemistry is an excellent means for providing an early warning of potentially damaging changes in stressed fish. Normal ranges of plasma activity vary between species and normal values must be produced from a representative population of each species. However, in many instances, these values in gamefish will be based on small sample sizes (<10) (*5, 9, 10*). Serum alkaline phosphatase activity is positively correlated to hepatic lesions in yellowtail tuna (*Seriola lalandei*) (*4*). In plasma of two examined specimens (No. 2 and 6) we observed higher levels of both AST and ALT when compared to other tuna in assay. It is difficult to determine when to consider a value »elevated« since normal ranges of tuna plasma values are not available. However, yellowfin tuna (5) demonstrated higher mean AST and ALT values (390 + 211 U/L and 61 + 77 U/L, respectively) com-

pared to our data. Elevated AST and ALT levels might be due to damage to the liver, but other organs might also be affected, like the kidney and gills. The increased AST and ALT activity in fish may reveal possible leakage of enzymes across increasingly semi-permeable plasma membranes and/or the increased synthesis of enzymes by the liver (*11*). It is possible that such conclusion could be made for specimen No. 6.

GGT is an enzyme considered to be a specific diagnostic indicator of cholestatic disease in mammals (*8*). In this study, tuna plasma GGT was not detected, which is consistent to a previous study (*5*) on eight species of big gamefish where authors could not establish detectable levels of GGT in plasma.

Typically, blood glucose levels taken from sharks at the time of their capture were reported to range from 3.3–5 mmol l^{-1} (*12*). GLU values in diploid and triploid Atlantic salmon (*Salmo salar*), subjected to confinement stress varied from 3.3–5.3 mmol l^{-1} (*13*). Secondary responses to stress (ventilation frequency, heart rate, hematological parameters, blood glucose) range in their responsiveness to different stimuli. Accordingly, low intensity stress did not alter GLU levels of the rainbow trout (*Oncorhynchus mykiss*), and were maintained at 3 mmol/l (*14*). In white sturgeon (*Acipenser transmontanus*), mean GLU levels in normally fed fish were constant at 6.5 mmol l^{-1} (*15*). The capturing of red drum (*Sciaenops ocellatus*) with a dip net, exposing them to air for 2 minutes, and transferring them to another tank elicited marked elevations of GLU levels from <3 mmol l^{-1} to 12.5 mmol l^{-1} (*16*). Mean GLU value recorded in this study was 10.58 mmol l^{-1}; however, for yellowfin and skipjack tuna, GLU values (*5*) were 4 and 5.5 mmol l^{-1} respectively. It is known that plasma GLU and CHOL concentrations may also be abnormally raised after recent meal. Tuna in this assay were not fed one day prior to sampling so that higher GLU values, when compared to other reported species, might be associated with intensified physical activity prior to harvesting, an acute stress event.

Our CHOL and TRIG data did not vary significantly from reference values for captive pink snapper (*Pagrus auratus*) (*17*). However, normal plasma cholesterol values in most marine fish range from 2.22–23.8 mmol l^{-1} (*18*). Since TRIG are mainly synthesized in the liver, form carbohydrates providing a secondary energy source and are stored in fatty tissue, they indicate acute liver disease and a high fat diet. The use of oily baitfish as a source of food for captive tuna poses a number of problems, among which is the presence of thiaminases and oxidized lipids in these fish, which are likely to be responsible for nutritional problems in the tuna (*19*).

Tuna CK values corresponded identically to pink snapper CK reference values (1291 U/L), although biochemical results for CK and AST were extremely variable in the snapper (also demonstrated by our data), which was considered a direct result of muscle damage during collection (*17*). Since the transportation of examined tuna was done six months prior to sampling, any muscle damage (cardiac or skeletal) during that operation should have healed. It is therefore tempting to believe that high levels of CK could be a result of muscle lesions or vigorous exercise of fish during harvest, since CK is the major source of high-energy phosphate used in muscle contraction.

Tuna LDH mean was much higher than the means reported for freshwater tilapia (*Oreochromis niloticus*) or red pacu (*Piaractus brachypomus*), which did not exceed 240 U/l (*20, 21*), and also higher than LDH mean of sandbar sharks (*Carcharhinus plumbeus*) (106 U l^{-1}) (*10*). Increased levels could be connected with hepatic necrosis, hepatic parenchimal lesions, fatty liver, skeletal muscle lesions, myocardial infarction or tumors. This partially correlates with histological findings on the livers of examined tuna, which demonstrated fatty changes. It is known that the enzymes found in the cellular cytoplasm or mitochondria are ALT, AST, CK and LDH. These enzymes are released from the cells during occurrence of necrosis. Plasma activity is therefore a function of the rate at which the enzyme is being released from the cells and the rate at which it is cleared from circulation. When only minor, transient changes occur in the morphology and function of cells, these enzymes may be released quite readily. They are therefore early indicators of increased cellular semi-permeability (*22*). Indeed, most hepatic lesions were histologically determined in specimens with the levels of LDH, CK and AST higher than in other specimens (No. 2, 5, 6).

Plasma protein levels were found to increase with age in juvenile hybrid striped bass (*Morone chrysops x Morone saxatilis*) (*23*). To some degree it could be attributed to ALB fraction, since albumin constitutes the major single component in plasma TP. Similar elevations over time could also be expected for tuna. Increased concentrations of TP in fish can be caused by structural liver alterations, and can occur from the impaired control of fluid balance that accompanies strenuous exercise, while decreased concentrations may occur due to a failure of protein synthesis, for example starvation (*24*). TP concentration was found to decrease as the histopathological organ indices of liver and gills increased (*24*). Measurement of serum or plasma albumin is of considerable diagnostic value in laboratory animals as it relates to general nutritional status, the integrity of the vascular system and liver function (*8*).

Urea values of tuna in our analyses were 10-fold lower than those for yellowfin tuna (*5*). Physiological/pathological conditions in which plasma URE concentration may be abnormally lowered are described as low protein diet or hepatic failure (*22*), which we find unlikely due to protein-rich diet and different degrees of fatty lesions within hepatocytes which are not of such intensity to demonstrate hepatic failure.

Baseline ranges of several clinical chemistry variables need to be established for stocks of bluefin and other

tunas of aquacultural importance in order to facilitate disease diagnostics and health evaluation in these species. Values provided here, with other studies to follow, will be useful for establishing those relationships.

***Acknowledgements**: The authors wish to thank Mr. Mladen Milaković, the owner of the tuna farm, and Mr. Goran Brstilo, the principal diver, for their help and assistance in providing tuna for examinations. We acknowledge the assistance of Dr. Zoran Lipej and Dr. Emil Srebočan in the processing of samples.*

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