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Biochemical blood parameters: are they effective biomarkers of monitoring fish condition?

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Abstract

The use of biomarkers has become attractive and useful for monitoring environmental quality and health of fish inhabiting polluted ecosystems. Among them, plasmatic biochemical parameters could be a promissory indicator of fish health condition. The question that arises is if these parameters can be applied to chronic toxicity. In this study we investigated the use of plasmatic biochemical parameters as indicators of fish chronic exposure to metals.

Mullets (*Liza saliens*), inhabiting a metal polluted habitat, a small coastal lagoon Esmoriz-Paramos (Northwest coast, Portugal), showed alterations of plasmatic parameters related with Cu and Zn bioaccumulation in tissues. The effect of metals chronic exposure in plasmatic parameters is scarcely studied and this study in a lagoon, where fish are exposed to metals for its life span, enabled a realistic long-term evaluation of the stress responses.

Keywords: blood parameters; biomarkers; chronic toxicity

1. INTRODUCTION

Blood chemistry measurements can provide valuable physiological indices that can be useful indicators of sub-lethal effects in fish [1]. However, there is scarce information on the use of plasmatic parameters in the assessment of physiological stress arising from chronic exposure.

The habitat under research is Esmoriz-Paramos, a small polluted coastal lagoon (Northwest coast, Portugal), where mullets, *Liza saliens*, are exposed to heavy metals in water and sediments resulting in high accumulation of Cu and Zn in their tissues. The unique characteristics of this small lagoon, led to the fish permanency in the lagoon for its life span and in consequence to a long time of exposure [2]. Previous results demonstrated, that mullet has the ability to inhabit this polluted environment adjusting some metabolic mechanisms.

The question that comes up is if plasmatic biochemical levels could be a tool in a long-term evaluation of the fish stress responses.

Thus, the main goal of this study was to compare the plasma biochemical parameters from a lagoon mullet population with those of a control group, with the intent that this information would be useful to establish reference intervals to apply in future research of chronic stress conditions.

To avoid seasonal variations adult fish was sampled in April in the post-spawning period. A pool of 30 mullets was collected from the sea, 14 km northwards from the cost, using a gill net. Simultaneously 10 mullets from Esmoriz-Paramos lagoon were also collected. Fish was quickly anesthetized and blood was drawn from the caudal vessels with heparinised syringes. Plasma was obtained by centrifugation (5 min, 10000 g, 4°C) and frozen until analysis. Total body length and weight were recorded to calculate condition factor (K = body weight (g)/(fish length (cm))^3 x 100).

Plasma chemical analysis

Any haemolysed or insufficient volume samples were discarded. Plasma samples were analysed using an automated dry chemistry system (Clinical Diagnostics, VITROS 950). Glucose, total protein, albumin, calcium, chloride, potassium, sodium and phosphorus concentrations were determined as well as the enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

Globulins were calculated from the difference between total protein and albumin values. All tests conditions and reagents were standard for the equipment and obtained from VITROS Chemistry Products.

Glucose concentration was colorimetric determined by H_2O_2 formation, total protein was determined by the biuret reaction and albumin concentration was colorimetric determined by dye-binding method with bromocresol green. Sodium, chloride and potassium were measured by ion-selective electrodes. Inorganic phosphorus and calcium were measured using colorimetric methods, ammonium molybdate reaction and arsenazo (III) dye formation, respectively.

Enzyme activities were colorimetric determined, AST activity was measured by dye formation via hydrogen peroxide (H_2O_2), ALT activity was measured by the NADH oxidation rate and ALP was measured by the *p*-nitrofenol formation.

Data analysis

All statistical analyses were performed with SPSS statistical program. For each parameter descriptive statics were computed, median, maximum, minimum, coefficient of variation, mean and standard deviation. Man-Whitney test was employed to compare fish biochemical data in the two environments, within the same size class. Significance level of 0.05 was assumed throughout.

3. RESULTS

The size of mullets captured in the sea was 21.60 - 47.00 cm in length, with a mean of 28 cm. The condition factor (K) of these mullets ranged from 0.31 to 0.99 and the mean K was 0.73. Table 1 summarizes the values of plasma biochemical parameters obtained in the sea mullet population. Coefficients of variation (C.V.) are also shown as a measure of parameter variability.

For each parameter, all values were checked and outliers were discarded: one for sodium, chloride, and calcium and two for aspartate aminotransferase (AST).

Histograms of the plasma biochemical parameters are presented in Figure 1, to illustrate the distribution pattern.

Table 1. Descriptive statistics of plasma biochemical values of control mullets (from the sea).

Biochemical Parameters	*Median*	*Mean ± SD*	*Minimum*	*Maximum*	*C.V.*
Albumin (g/dL)	1.00	1.39 ± 1.05	0.40	4.00	75
Total protein (g/dL)	3.20	3.80 ± 1.94	1.00	8.30	51
Globulins (g/dL)	2.20	2.51 ± 0.97	0.60	4.40	39
Glucose (mg/dL)	85.00	88.07 ± 49.95	10.00	199.00	57
Calcium (mg/L)	6.15	5.94 ± 1.09	3.80	7.40	18
Phosphorus (mg/dL)	14.20	13.64 ± 4.06	5.80	19.00	30
Chloride (mEq/L)	157.00	158.07 ± 9.93	141.90	176.60	6
Potassium (mEq/L)	7.80	7.31 ± 2.89	2.70	12.40	40
Sodium (mEq/L)	188.25	186.97 ± 16.44	162.40	226.80	9
AST (U/L)	111.00	142.91 ± 84.08	34.00	306.00	59
ALT (U/L)	19.00	21.96 ± 19.12	3.00	78.00	81
ALP (U/L)	23.50	38.38 ± 32.11	10.00	98.00	84

C.V, coefficient of variation = standard deviation/mean x 100.

Plasma biochemical values obtained in mullets captured in the Esmoriz-Paramos lagoon are presented in Table 2 and compared to control intervals. Number of mullets with values out of the control range (Table 1), are also shown.

Table 2. Descriptive statistics of plasma biochemical values of mullets from the lagoon, N=10.

Biochemical Parameters	*Median*	*Mean ± SD*	*Minimum*	*Maximum*	*C.V.*	*Out*
Albumin (g/dL)	2.15	2.72 ± 1.51	1.10	6.50	56	2
Total protein (g/dL)	5.65	6.45 ± 2.30	3.90	12.00	36	0
Globulins (g/dL)	3.55	3.73 ± 0.81	2.80	5.50	22	2
Glucose (mg/dL)	195.00	185.30 ± 82.45	26.00	345.00	45	3
Calcium (mg/L)	7.30	7.05 ± 0.98	5.30	8.20	14	5
Phosphorus [b] (mg/dL)	15.30	15.88 ± 2.08	12.30	18.50	13	0
Chloride (mEq/L)	128.05	129.66 ± 7.55	119.40	148.10	6	9
Potassium [b] (mEq/L)	5.45	5.18 ± 1.44	2.90	6.90	28	0
Sodium (mEq/L)	153.40	152.28 ± 9.11	135.60	168.40	6	9
AST (U/L)	202.50	255.10 ± 132.08	63.00	454.00	52	3
ALT [b] (U/L)	3.00	4.13 ± 2.47	3.00	10.00	60	0
ALP [a] (U/L)	28.00	32.44 ± 16.55	10.00	62.00	51	0

a, N= 9; b, N= 8; C.V, coefficient of variation = standard deviation/mean x 100.

Lagoon mullets showed an average length of 37.72 cm, with a range 26.50-44.60 cm, and an average *K* of 0.95, range 0.75 -1.19. They also presented an average concentration of metals in liver of 283 mg $Cu.kg^{-1}$ and 33 mg $Zn.kg^{-1}$, with a range 53- 464 mg $Cu.kg^{-1}$, and 63 – 190 mg $Zn.kg^{-1}$.

Some plasma biochemical values in lagoon mullets showed lower coefficients of variation than the control values: ALP (33% lower), ALT (21% lower), phosphorus (17% lower), albumin (19% lower) and total protein (15% lower), with consequent same pattern in globulins. Nevertheless, the remaining parameters showed higher variations than the respective control values.

ALT activity, chloride, potassium and sodium average values were lower in lagoon mullets than in the control mullets. The remaining parameters showed higher average values, except ALP activity, that was similar in the two environments. The same trend was observed for the median values of each parameter.

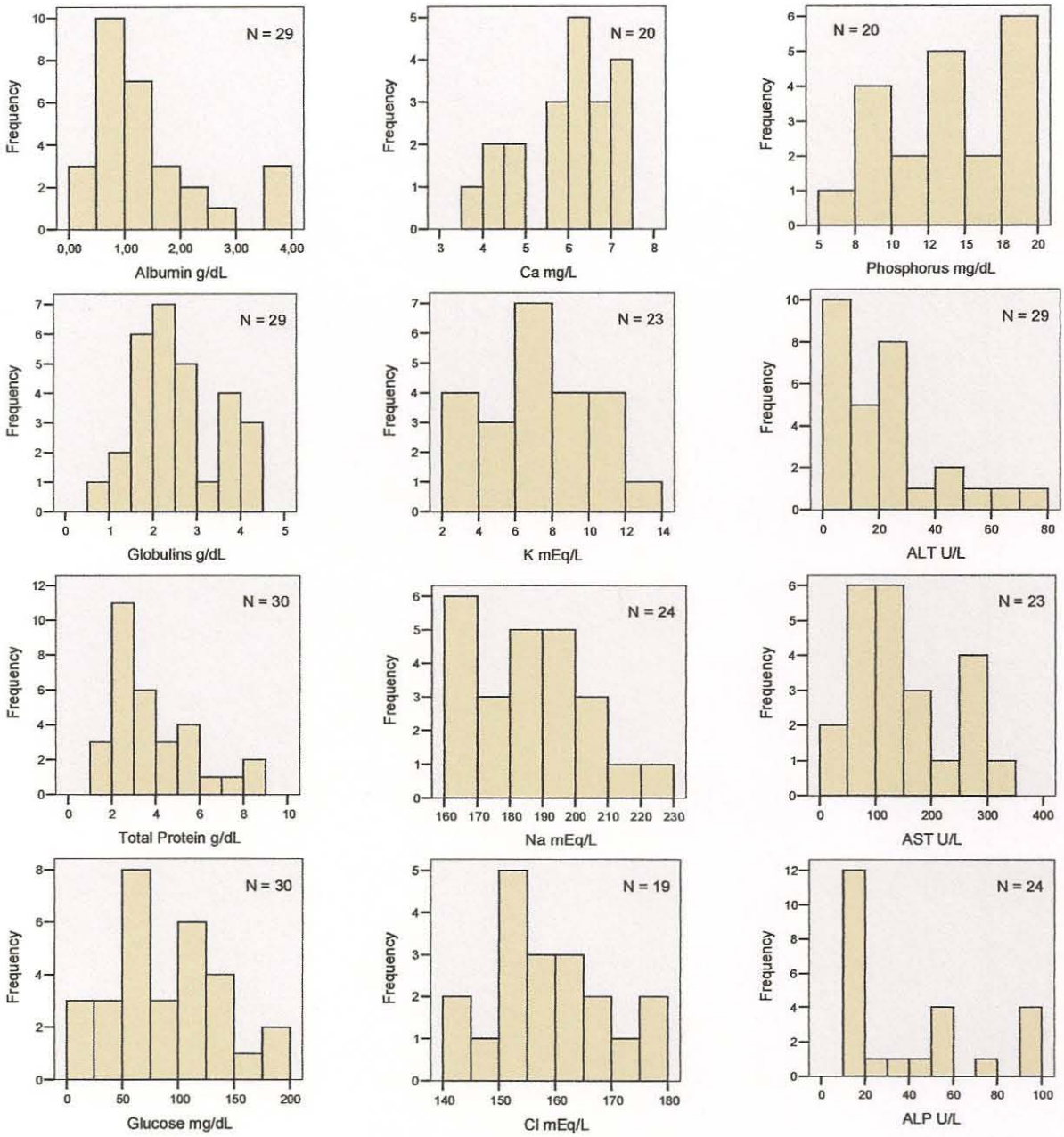

Figure 1. Histograms of distribution of biochemical parameters of control mullets.

Yet, the majority of lagoon mullets showed sodium and chloride (90%) lower values than the intervals obtained for sea mullets. Also we observed that 50% of calcium values, 30% of AST and glucose values, 20% of albumin and globulins values were higher in lagoon mullets.
Even when plasma parameters were compared within the same size class, lagoon mullets still showed the same tendency. However, besides high *K,* lagoon mullets also showed high levels of total protein, whereas calcium values did not differ between the two environments.

4. DISCUSSION

Plasma biochemical values are important parameters for assessing animal health and the reliability of laboratory results. Although reference values of plasmatic parameters for some fish species have been published, most studies use the mean and standard deviation to establish the reference intervals [1,3], which is not in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). In order to establish a reference interval the NCCLS typically requires a minimum of 120 normal individuals [4]. Recommendations to establish

reference parameters for plasma chemistry advise the use of the nonparametric rank percentile method. Though this method is valid regardless of data distribution, results can be misleading if the number of samples is less than 50.
The purpose of this study was to compare plasmatic biochemical parameters of a control reference group of adult mullets with those of mullets under chronic metal exposure, with the intent of getting valuable information that can be applied in other polluted environments to assess metal induced stress.
Evaluation of health status requires comparison with known healthy individuals. However biochemical values for mullets are scarce. In this study, mullets captured in open sea were used as controls and mullets chronically exposed to metals were collected from a small polluted coastal lagoon, where fish remains for all its life. Thus, the question that arises is if plasmatic biochemical parameters, in fish chronically stressed, exhibit any difference relative to the reference population.

The interactions of age and gender, among others, on these biochemical parameters have been documented. Some authors state, that major biochemical fluctuations occur in pre-spawning period [5,6]. In this study, the variation due to reproductive stage was avoided since fish capture occurred simultaneously in the two environments, in post-spawning period, when undeveloped gonads were observed disallowing gender differentiation.
It has been reported that mullet plasma enzymes, ALP, ALT and AST, are the parameters with highest variation when compared with the other parameters [5]. In agreement with these findings, the highest variability found in this study was for serum enzymes and the lowest variability was recorded for electrolytes.
Comparisons between the same size classes are preferable since age-induced variability can occur and our observations support the general idea that physiological and nutritional aspects may play an important role in the levels of these plasma biochemical parameters. As with any integrative and relatively non-specific parameter, condition factor (K) is affected by many factors and the high K found in lagoon mullets seem to be related to nutritional factors [7].

Glucose concentration in blood is a sensitive indicator of fish stress and some studies concluded that metals can induce hyperglycemia in different fish species [8]. In agreement with those reports, glucose levels in lagoon mullets were higher than in sea mullets. This can be an indication of metals induced effects. Protein levels, albumin and globulins were also increased in lagoon mullets compared to controls. Increased albumin could be related to its function in blood pressure maintenance, as well as its function as biomolecules transporter including toxics. High globulin and protein levels could be the result of chronic inflammatory conditions, by both infectious and non-infectious causes.

Exposure of fish to heavy metals may result in primary toxic effects on the branchial epithelium [9] resulting in various degrees of ion regulatory disruption [10,11,12]. Since gills are the major sites of osmotic and ionic regulation in fish, any changes in gill morphology may also result in altered osmotic and ionic status. The majority of lagoon mullets have sodium and chloride levels decreased. Although the mechanisms of heavy metals toxicity are well known in acute exposure, the process may differ in chronic exposure. It would be expected that mullets, after spending several years in the lagoon, have attained a sort of ionic homeostatic disturbances. The mechanisms of acute copper toxicity include osmoregulatory disturbances involving sodium, chloride and potassium gill uptake [10,11]. Gill epithelium of *Liza saliens* showed different histological changes, concomitant with high metal levels [2,13] that certainly affected plasma ions levels.

The increased plasmatic AST activity in lagoon mullets, as already shown in previous studies, together with the present results, reinforce the idea of a change in protein metabolism, rather than a consequence of cell permeability and integrity alterations [14].

In conclusion, this study shows that, although the long-time exposure of lagoon mullets to stress conditions, induced-plasma biochemical changes occur, that can be used as a tool to monitor fish condition. Even so, caution must be taken since the establishment of accurate reference intervals requires a large size sample, specially if they are to be established based on age classes, gender or other grouping criteria.

References

1. Ekrem, A.Ç., 2004. Blood chemistry (electrolytes, lipoproteins and enzymes) values of black scorpion fish (*Scorpaena porcus* L., 1758) in the Dardanelles, Turkey. *J. Biological Scienc.*, **4(6)**, 716-719.
2. Fernandes, C., Fontaínhas-Fernandes, A, Monteiro, S.M., Salgado, M.A., 2007. Histopathological gill changes in wild leaping grey mullet (*Liza saliens)* from the Esmoriz-Paramos coastal lagoon, Portugal. *Environmental Toxicology*, **22 (4)**, 443-448.
3. Hrubec, T.C., Cardinale, J.L., Smith S. A., 2000. Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis* hybrid). Vet. Clinical Pathology, **29 (1)**, 7-12.
4. NCCLS, 2000. How to define and determine reference intervals in the clinical laboratory: approved guideline. Doc. C28-A2 USA.
5. Folmar, L.C., Moody, T., Bonomelli, S., Gibson J., 1992. Annual cycle of blood chemistry parameters in striped mullet (*Mugil cephalus* L.) and pinfish (*Lagodon rhomboids* L.) from the Gulf of Mexico. *J. Fish Biol.*, **41**, 999-1011.
6. Mercaldo-Allen, R., Dawson, M.A., Kuropat, C.A., Kapareiko, D., 2003. Variability in blood chemistry of yellowtail flounder, *Limanda ferruginea*, with regard to sex, season, and geographic location. NOAA Technical Memorandum NMFS-NE-**180**, 1-20.
7. Fernandes, C., Salgado, M.A.,.Fontaínhas-Fernandes, A., 2008. *Metabolic Mechanisms of Adaptation to Chronic Metal Exposure – A Case Studty*. In Heavy Metal Pollution. Ed Samuel E. Brown and William C. Welton. Nova Science Publishers, Inc.
8. Varanka, Z., Rojik, I., Varanka, I., Nemcsók, J., Ábrahám, M., 2001. Biochemical and morphological changes in carp (*Cyprinus carpio* L.) liver following exposure to copper sulfate and tannic acid. *Comp. Biochem. Physiol.*, **128**, 467-478.
9. Playle, R.C., Gensemer, R.W., Dixon, D.G., 1992. Copper accumulation on gills of fathead minnows: influence of water hardness, complexation and pH of the gill micro-environment. *Environ. Toicol. Chem.*, **11**, 381-391.
10. Grosell, M., Wood, C.M., Walsh, P.J., 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. *Comp Biochem Physiol Part C* **135**, 179-190.
11. Mazon, A.F., Monteiro, E.A.S., Pinheiro, G.H.D., Fernandes, M.N., 2002. Hematological and physiological changes induced by short-term exposure to copper in the freshwater fish, *Prochilodus scrofa. Brazil J Biol* **62(4A)**, 621-631.
12. Taylor, L.N., McFarlane, W.J., Pyle, G.G., Couture, P., McDonald, D.G., 2004. Use of performance indicators in evaluating chronic metal exposure in wild yellow perch (*Perca flavenscens*). *Aquat Toxicol* **67**, 371-385.
13. Fernandes, C., Fontaínhas-Fernandes, A, Monteiro, S.M., Salgado, M.A., 2007. Changes in plasma electrolytes and gill histopathology in wild *Liza saliens* from the Esmoriz-Paramos coastal lagoon, Portugal. *Bulletin of Environ. Contamination and Toxicology*, **79 (3)**, 301-305.
14. Fernandes, C., Fontaínhas-Fernandes, A, Rocha, E., Salgado, M.A., 2008. Monitoring pollution in Esmoriz-Paramos lagoon, Portugal: Liver histological and biochemical effects in *Liza saliens*. *Environ Monit and Assessment,* **145**, 315-322.