Analysis of Neotropical Serum Fishes Esterases Activities for the Selection of Noninvasive Enzymatic Biomarkers

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Abstract

Enzymatic biomarkers can be effective tools for monitoring the health of aquatic organisms exposed to xenobiotics. The use of sample blood is beneficial to preservation, since it allows evaluations without the euthanasia of the animal, preserving the fauna and the balance of the ecosystem. Nevertheless, it is previously necessary to understand the kinetics of the enzymes and to determine their basal activity levels for the proper choice of the enzyme to be used for biomarking. The best assay conditions of carboxylesterase activity (CaE) present in the serum of four different species of neotropical fish were established and their activities were compared with other blood esterases. Also, the CaE were measured at summer and winter to verify possible seasonal changes. The CaE was assayed with the substrate para-nitrophenylacetate (p-NPA) by the appearance of product in one minute reaction. The dourado CaE had a KM and Vmax value similar to that of pacu, but the curimbatá showed the best catalytic efficiency to degrade p-NPA. In piauçu the activity of serum CaE is not as efficient when compared to the other species. Dourado, pacu and curimbatá have higher CaE than cholinesterase activity, distinct of piauçu. The CaE of pacu serum decreases in the winter. Thus, it is shown to be important to establish the level of activity of each esterase per species and to verify if seasonal variation may influence the analysis in the monitoring of enzymatic biomarkers.

Keywords: Carboxylesterase; Cholinesterase; Enzymes; Blood

Introduction

Assessments of pollutants effects on organisms aim to estimate how intensely anthropogenic intervention can occur without greatly altering the ecological balance of an area. Samples obtained from animals, as tissues, cells or biological fluids [1] are useful for assessing and controlling the risk associated with long-term exposure to xenobiotics, such as heavy metals, hydrocarbons and agrochemicals [2].

The biomarkers compare a normal biological process with the value obtained in a pathogenic or stress state. Since the enzymatic reactions can show variations, it is possible to obtain information about the health of the animals and the environment that they inhabit through tissue samples. The enzymatic biomarkers are interesting in monitoring because they may reflect sublethal responses, indicating early damage before tissue lesions are established. Procedures to found early warning signals that reflect adverse biological responses due to anthropogenic changes stand out in researches for enabling actions prior to extreme environmental damage [3].

Esterases are inhibited by phosphates, therefore cholinesterases has been widely used to evaluate the harmful effects of pesticides and are widespread as fish biomarkers of exposure because they affinity to organophosphates

and carbamates compounds [4-6]. Liver, muscle and brain samples are frequently used for enzymatic analysis of cholinesterase. Unfortunately, holding these samples often implies the death of the specimen, thus the use of blood sample seems to be a better procedure.

The kinetics of blood enzymes of neotropical fish species it is not well known. Some studies have already shown that fishes have biochemical mechanisms, involving the inhibition of esterases, which protect them against exposure to organophosphates. Salles, et al. [7] demonstrated that the Pantanal piauçu plasma activity of butyrylcholinesterase (BChE) is three times higher than human plasma and 34 times higher than tilapia plasma, and that this is the main esterase "capture" of organophosphates present in the plasma of piauçu. Cunha Bastos, et al. [8] verified that paraoxonase activity in dourado serum reached values six times higher than those found in pacu and cascudo serum, and 23 times higher than in serum of matrinxã.

Organophosphates can also bind irreversibly to another non-target esterases, for example the carboxylesterase (CaE). Such binding makes the CaE to act as a detoxification enzyme, because when it binds organophosphates it prevents an increase in the concentration of organophosphate at the site of the target enzyme, being able to alter the toxicity profile of some pesticides that inhibit cholinesterases [9]. According to Satoh T [10] wide variety of carboxylesterases found in tissues and plasma show greater promiscuity in substrates for which they catalyze, so the multiplicity of structure of these enzymes allows hydrolyzing a diverse range of esters. Hence, this esterase may be involved in protecting the organism against xenobiotics and being an interesting biomarker.

The characterizations of carboxylesterases are more related to insects and humans, and there are few studies with non-target organisms, such as fishes [11]. Dalzochio, et al. [12] studying biomarkers of aquatic environments in Brazil showed that the researches are concentrated in only two states, lacking reports on a Center-West and Northern region. Therefore, researches with neotropical fish are important to enrich biochemical knowledge and to understand the various adaptations found in these organisms. The aim of this study is to standardize and determine basal activity levels of esterases, focusing on carboxylesterase, in serum of four neotropical fish species at different seasons of the year as a subsidy for future studies with enzymatic activity biomarker in fish serum.

Material and Methods

Fishes and Obtaining Serum

Four specie of neotropical fishes were analyzed: the dourado *Salminus brasiliensis*, the pacu *Piaractus mesopotamicus*, the curimbatá *Prochilodus lineatus* and the piauçu *Leporinus macrocephalus*.

The blood samples were obtained from adults' fishes coming from farming. The biometric data are present in Table 1.

Species	Dourado	Pacu	Curimbatá	Piauçu
n	42	22	5	3
Weight (g)	1704 ± 611	233 ± 129	290 ± 119	414 ± 156
Total length (cm)	49.4 ± 5.9	22.58 ± 3.66	30.6± 1.4	26.5 ± 3.5

Table 1: Biometric data of species.

Blood was collected by puncture of a vessel from the caudal peduncle. The blood was let in an assay tube on ice to clot. Clotted blood was centrifuged at 3500 rpm for 10 minutes. The straw-yellowish supernatant serum was kept frozen until analysis.

Serum Esterases Enzyme Assays

Carboxylesterase activity (CaE) was carried out with para-nitrophenyl acetate (p-NPA) as substrate diluted in acetonitrile 100% according to Morgan, et al. [13]. The reaction medium was buffered with 0.1 M sodium phosphate solution, pH 7.7 at 25°C. The enzyme activity was measured by determining the appearance of para-nitrophenol for one minute at the wavelength of 400 ηm on a Shimadzu UV-1800 spectrophotometer. The molar absorptivity coefficient of p-nitrophenol used was 13,000 $M^{-1}.cm^{-1}$. For which specie were made a substrate standard curve to verify optimal assays conditions. The p-NPA substrate has spontaneous hydrolysis. To record only the catalyzed reaction, a blank was made consisting of the activity test without adding the sample. The enzymatic activity was obtained by subtracting the blank value from the value of each assay.

Cholinesterase activity (ChO) was conducted according to Ellman, et al. [14], using a Shimadzu UV-1800 spectrophotometer measuring continuously recorded up to one minute at 412 ηm. The reaction was carried out

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in buffer solution of sodium phosphate 0.1 M, pH 7.5, at 25°C, containing 1.87 mM ASCho or BSCho as substrates, in the presence of 0.32 mM DTNB. The molar absorptivity coefficient used for ChO was 14,150 $M^{-1}.cm^{-1}$.

Determination of K_M and Vmax and Statistical Analyses

The values of maximum velocity (Vmax) and apparent K_M were determined using the Michaelis–Menten equation with the GraphPad Prism software. All enzymatic activities were expressed as $\mu mol.min^{-1}.mL^{-1}$ of serum and the results are presented as mean ± standard deviation. A value of p <

0.05 was considered statistically significant.

Results

Standardization of Serum CaE Assay

Blood from at least three specimens was collected to obtain a serum pool to establish how the variation of p-NPA concentration affects the initial velocity reaction. The Michaelis-Menten curve was obtained for each species, as shown in the graphs of Figure 1. The results of the standardization also determined the Vmax for p-NPA, as well the apparent K_M value (Table 2).
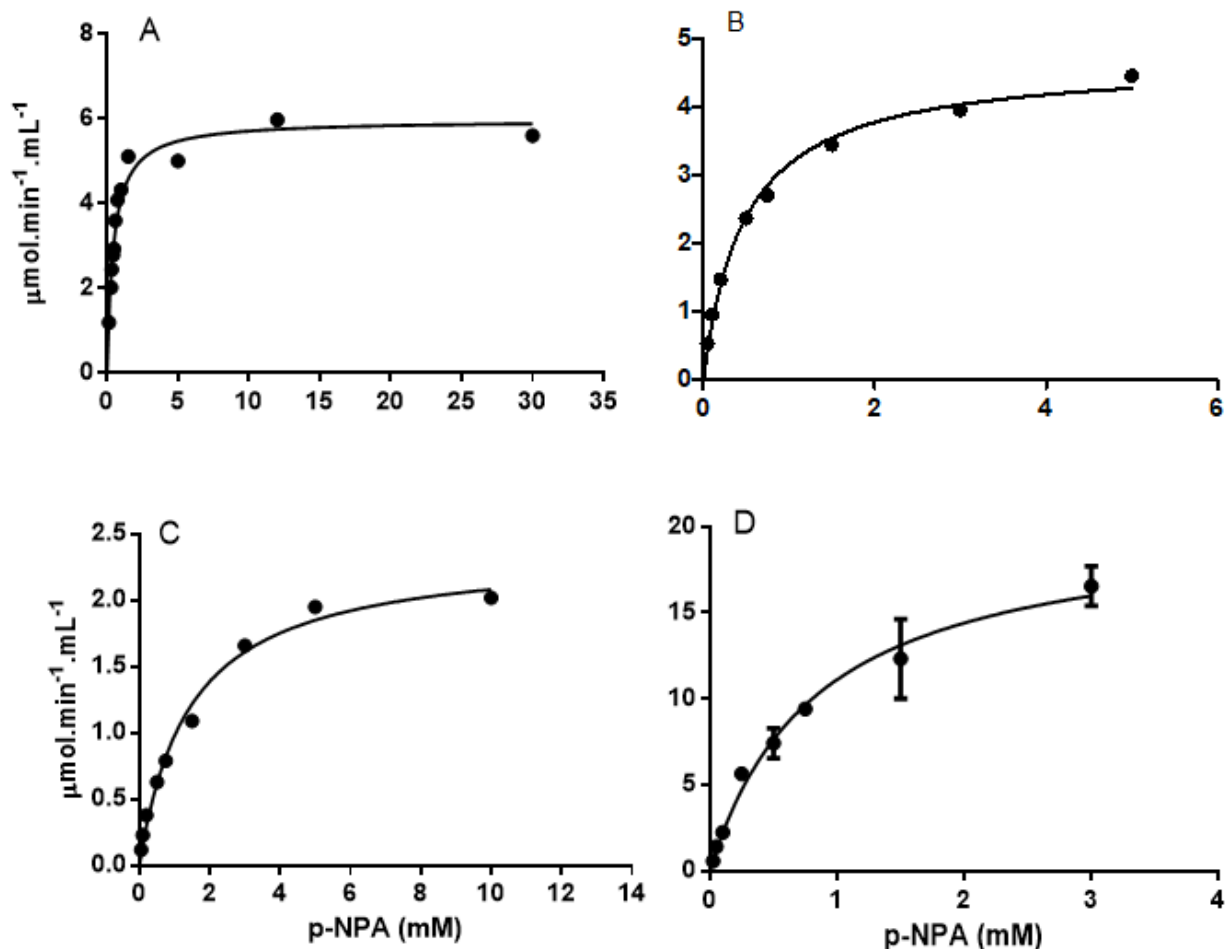

Figure 1: Effect of p-NPA on carboxylesterase activity in serum of neotropical fishes by the Michaelis-Menten curve. A) dourado, *Salminus brasiliensis* B) pacu, *Piaractus mesopotamicus* C) piauçu, *Leporinus macrocephalus D*) curimbatá, *Prochilodus lineatus*.

After determining the optimum assay conditions with the p-NPA substrate for each species, the CaE in the serum of each individual was assayed and the mean basal level is demonstrated in Table 3. Cholinesterases activities were also verified in the serum of the four species (Table 3).

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Comparison between Species

The values of K_M and Vmax varied between species (Table 2). The curimbatá presented the highest Vmax and the best catalytic efficiency, however, the K_M value is the second largest. The dourado and pacu had close values. The piauçu CaE serum activity is not as efficient when compared to the other fishes.

Species	CaE in serum		
	KM (mM)	Vmáx (µmol.min-1.mL-1)	Vmáx/KM k (min-1)
Dourado (*Salminus brasiliensis*)	0,41	5,73	13,97
Pacu (*Piaractus mesopotamicus*)	0,48	4,69	9,77
Curimbatá (*Prochilodus lineatus*)	0,84	20,45	24,34
Piauçu (*Leporinus macrocephalus*)	1,43	2,38	1,66

Table 2: Values of Vmax, apparent K_M and catalytic efficiency of p-NPA hydrolysis by neotropical fish's serum pool.

Comparing the value of CaE with ChO activities in fish serum, it is possible to verify that for each species there is a variation between the average activities measured for each enzyme (Table 3). The piauçu exhibits superior activity of BChE in relation to other esterases, whereas in dourado the BChE is almost undetectable. The dourado, pacu and curimbatá have in serum higher activity of CaE than ChO.

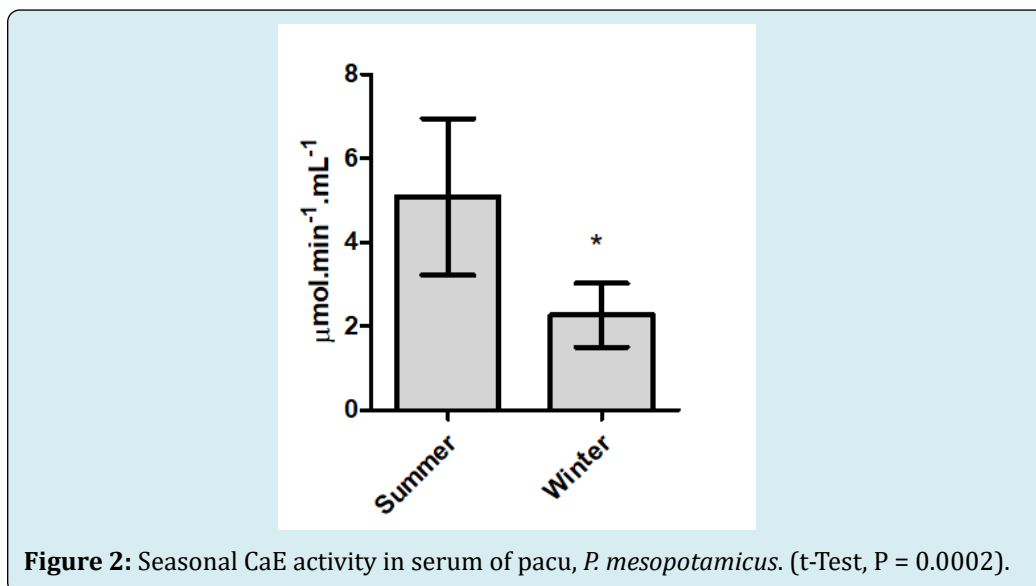| Serum	Enzymatic activity (µmol.min-1.mL-1)		
	AChE	BChE	CaE
Dourado	0,14 ± 0,04	0,01 ± 0,06	6,96 ± 3,71
Pacu	0,74 ± 0,18	1,03 ± 0,30	3,67 ± 2,00
Curimbatá	3,02 ± 1,69	5,85 ± 3,46	16,95 ± 1,08
Piauçu	9,35 ± 4,92*	21,72 ± 11,74*	1,56 ± 0,29

*Reference Salles. et al. [7].
Table 3: Esterases basal enzymatic activities in serum of neotropical fishes.

Seasonal Comparison

For the dourado and pacu it was possible to collected blood during the winter and summer, in order to verify if there is a seasonal change. The pacu serum CaE have seasonal variation (Figure 2), with higher activity in summer (5.08 ± 2.26 µmol.min^{-1}.mL^{-1}) than in winter (1.86 ± 0.75 µmol .min^{-1}. mL^{-1}).

Figure 2: Seasonal CaE activity in serum of pacu, *P. mesopotamicus*. (t-Test, P = 0.0002).

Although the dourado CaE serum also tended to decrease during the winter, no statistically significant difference was observed for the average activity (Figure 3).
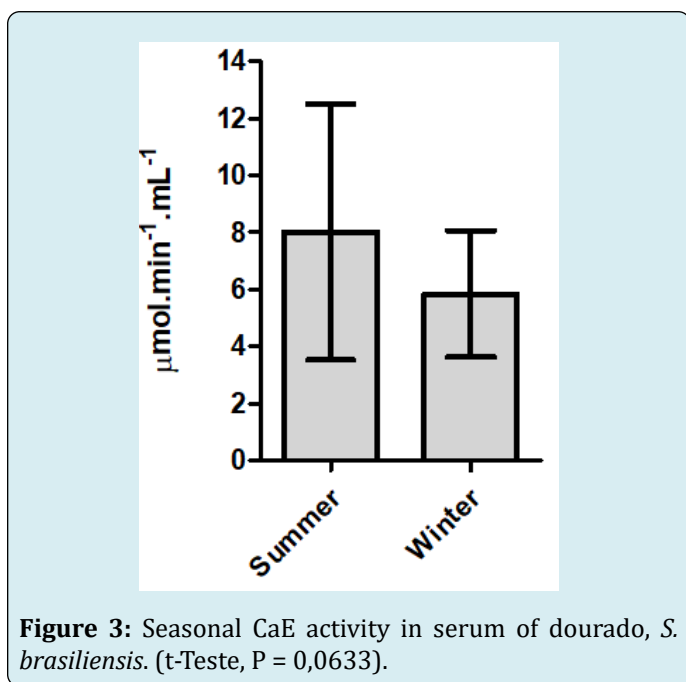

Figure 3: Seasonal CaE activity in serum of dourado, *S. brasiliensis.* (t-Teste, P = 0,0633).

Discussion

The hydrolysis of p-NPA was detected in the serum of the four neotropical fish species evaluated. The assay of CaE activity has desirable characteristics for a biomarker, since it is fast to analyze, have low cost and can be measured in serum. Wheelock, et al. [17] reported that the general CaE activity of colorimetric assays using substrates such as p-NPA or α-naphthyl acetate are convenient because of their easy availability and relatively inexpensive optical assay required for data acquisition. However, the authors point out that some CaE isoenzymes do not hydrolyses these substrates efficiently.

The use of serum has proved to be practical for assaying enzymatic biomarkers, being CaE and BChE the better detection activities. An expressive advantage of these biomarkers is that the assays preserves the animal life, in contrast to the use of AChE activity in muscle or brain. The analysis of CaE from the blood of the fish presents as a viable and a less invasive method. According to Thompson, et al. [18] plasma carboxylesterase activity is generally low in mammals, but is higher in fish, amphibians and birds.

Among the studied species, referent to CaE, curimbatá has the best catalytic efficiency, nevertheless the Vmax is reached in contact with higher p-NPA concentrations. The dourado and pacu have the lowest K_M, therefore reaching their maximum enzymatic efficiency with smaller amounts of substrate in the reaction medium. In rats, a study by Maxwell, et al. [15] involving CaE and reactions with organophosphorus compounds has shown that CaE appears to perform the role of a high-affinity/low-capacity detoxication process and this high CaE affinity is important for detoxication of very toxic organophosphates compounds that achieve lower *in vivo* concentrations. Thus, having a prompt and efficient catalytic activity of CaE in serum indicates that the species can capture stoichiometrically or hydrolyze more xenobiotics, avoiding to inhibit essential cholinesterases for the organism if they reach vital organs such as the heart and the brain.

Wheelock, et al. [19] testing the interaction of esterases and organophosphates in salmon tissues, detailed that understanding the change in the level of CaE, this activity can be used as biomarkers of fish exposure to agrochemicals. Among the species of neotropical fish evaluated, the curimbatá is probably the animal that is more resistant to contamination by organophosphates due to the higher CaE serum activity. However, piauçu has elevate serum BChE, which also gives it the ability to hydrolysis organophosphates efficiently.

Salles, et al. [7] demonstrated that the activity of AChE and BChE in serum of several fish species varies considerably, a fact corroborated by the results obtained with the serum CaE in the present study. Solé, et al. [20] characterizing different tissues esterases of a flatfish (*Solea senegalensis*) also found that in adult plasma the largest esterase activity is CaE. Thus, it is important to establish the basal level activity of each blood esterase per species, to choose the enzymatic biomarker that will best complete the desired monitoring.

The activity of pacu and dourado serum CaE has tended to decrease in the winter, therefore it is possible that during the winter species of neotropical fish are more susceptible to xenobiotic effects than in the summer. Dalzochio, et al. [12] compiled information on the use of biomarkers to assess the health of aquatic ecosystems in Brazil. Among their findings, they point out that the evaluation of more than one sampling site at different seasons may support in the interpretation of biomarkers responses. Solé, et al. [20] analyzed samples of goby sand fish (*Pomatoschistus minutus*) for one year to select biomarkers. The authors noted fluctuations in enzymatic activity, some of which are considered to be normal seasonal variations due to breeding period, but other changes may be due to local pollution inputs.

The enzymatic activities analyzed in the serum were important to establish the basal level for four neotropical fish species and to demonstrate that seasonal differences may occur. The results obtained are useful for inferring future analyzes, as well as for assisting in the plan of aquatic environments monitoring.

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