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## Cholinesterase Activity in the Head of Wild *Poecilia reticulata* from Bahia, Brazil: Biochemical Characterization, Effects of Sample Storage and Normal Range of Activity

C. STRINGUETTI,<sup>1</sup> L. GUILHERMINO<sup>2</sup> & E. M. DA SILVA<sup>1\*</sup>

<sup>1</sup>Institute of Biology, UFBA, Campus de Ondina, CEP 40170-115, Salvador, BA, Brazil

<sup>2</sup>Centro Interdisciplinar de Investigação Marinha e Ambiental (CIMAR), Rua dos Bragas, 177, CEP 4050-123, Porto, Portugal

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### ABSTRACT

In this study, the soluble cholinesterases (ChE) from the head of *Poecilia reticulata* from a wild population of Bahia, Brazil were characterized using different substrates (acetylthiocholine, butyrylthiocholine and propionylthiocholine) and selective inhibitors ( eserine sulphate, iso-OMPA and BW284C51). Possible effects of time (1, 2, 3, and 4 weeks) and different storage temperatures (freezer storage at *ca.*  $-20^{\circ}\text{C}$  and  $-50^{\circ}\text{C}$ , and liquid nitrogen system at  $-196^{\circ}\text{C}$ ) on ChE activity were also investigated, together with the normal range of ChE activity of non-exposed individuals. The results for the enzymatic characterization indicate that the enzyme present in the head of *P. reticulata* was mainly acetylcholinesterase. The mean and standard deviation of activity found in non-exposed wild males collected in different periods of the year were  $149.71 \pm 7.72$  SD U/mg protein, respectively. ChE activity significantly decreased after the seventh day of sample storage, independently of the temperature.

**Key words:** biomarker, cholinesterase, *Poecilia reticulata*, sample storage conditions.

### RESUMO

#### Atividade da colinesterase na cabeça de indivíduos selvagens de *Poecilia reticulata* (Bahia, Brasil): caracterização bioquímica, efeitos sobre armazenamento de amostras e atividade normal

Neste estudo foram caracterizadas as colinesterases solúveis (ChE) da cabeça de uma população selvagem de *Poecilia reticulata* do Estado da Bahia (Brasil) usando-se diferentes substratos (acetil-tiocolina, butiril-tiocolina e propionil-tiocolina) e seus inibidores seletivos (sulfato de eserina, iso-OMPA e BW284C51). Efeitos resultantes do armazenamento (1, 2, 3 e 4 semanas) e de diferentes temperaturas de armazenamento (em congelador *ca.*  $-20^{\circ}\text{C}$  e  $-50^{\circ}\text{C}$ , e em imersão em nitrogênio líquido, *ca.*  $-196^{\circ}\text{C}$ ) sobre a atividade enzimática da ChE foram também investigados, junto com a atividade enzimática normal de indivíduos não expostos. A média e o desvio-padrão da atividade normal de indivíduos machos não expostos e coletados periodicamente do campo foram de  $149,71 \pm 7,72$  DP U/mg protein, respectivamente. Os resultados para a caracterização enzimática indicam que a enzima presente na cabeça de *P. reticulata* foi, majoritariamente, a acetio-colinesterase. A atividade enzimática decresceu significativamente após o sétimo dia de armazenamento, independente da temperatura.

**Palavras-chave:** biomarcador, colinesterase, *Poecilia reticulata*, condições de armazenamento das amostras.

\*Corresponding author: Eduardo Mendes da Silva, e-mail: dasilva@ufba.br.

## INTRODUCTION

The inhibition of cholinesterase (ChE) activity has been largely used to diagnose the exposure of wild populations to anticholinesterase chemicals such as organophosphates and carbamate pesticides (Ozmen *et al.*, 1999; Fossi *et al.*, 2001; Fulton & Key, 2001). Usually cholinesterases are divided in acetylcholinesterase (AChE) and pseudocholinesterase or butyrylcholinesterase (PChE) (Fossi & Leonzio, 1994), according to their behaviour towards different substrates and selective inhibitors (Stojan *et al.*, 1998). Typical AChEs, such as mammalian ones, show preference for acetylthiocholine as substrate, relatively to butyrylthiocholine and propionylthiocholine, are strongly inhibited by low concentrations of BW284C51 and show low sensitivity to N,N'-diisopropylphosphorodiamic acid (Iso-OMPA) (Eto, 1974). Since different forms of ChE may have different sensitivity to anticholinesterasic contaminants and this may be a source of variation in ecotoxicological studies (Galgani & Bocquené, 1990; Garcia *et al.*, 2000), it is important to characterise the forms present in the population and in the tissues of the organisms to be studied before using these enzymes as biomarker (Garcia *et al.*, 2000). This is particularly important in biomonitoring programs with wild populations.

When dealing with a high number of samples, frequently it is not possible to perform ChE determinations immediately after the tissue isolation and they need to be stored before being analysed. Several authors report a significant decrease of ChE activity in samples of different species frozen for more than two weeks (Galgani & Bocquené, 1990; Day & Scott, 1990) while others did not found significant differences between fresh and frozen samples (Guilhermino *et al.*, 1996b). Since this variation may be species dependent and due to its importance, it is recommendable to choose the appropriated method and temperature of storage before performing ChE based studies requiring storage of samples.

*Poecilia reticulata* is an abundant species in Brazilian freshwater systems and since it is a recommended organism for aquatic toxicity testing in international protocols (OECD, 1993) it seems to be a good candidate to be used as bioindicator in biomonitoring studies. Therefore, the central objective of this work was to establish the basic conditions required for the use of wild *P. reticulata* populations to assess environmental contamination of freshwater ecosystems in Bahia (Brazil), by anticholinesterase pesticides. Specifically, the objectives of this work were: i) to perform the biochemical characterization of the soluble ChE present in wild *P. reticulata* head homogenates, (ii) to investigate the effects of time and temperature of preservation on ChE activity, and (iii) to determine the range of normal activity in fish from wild populations.

## MATERIALS AND METHODS

### Fish

Adult male fish (1.8-2.7 cm long) were collected from a non-contaminated site from the Capivari Creek (Cruz das Almas, BA, Brazil) and transported to laboratory in low density in 20 L

plastic containers to minimise stress. Organisms were held in 10-L flow-through aquaria containing natural glass-fibre filtered water from the collection site with approximately 20 individuals per tank, and fed *ad libitum*, once a day, with live cladocerans, supplemented with dried fish food (Alcon Basic). Vessels were cleaned and debris removed via vacuum suction every 2 d. They were acclimated to a temperature of  $26 \pm 2^\circ\text{C}$  and a photoperiod of 16:8 h light:dark periods, for at least 5 days in the laboratory prior to test initiation. The general health of the animals appeared good, and no evidence of fungal infection was observed during this period. In all experiments, adequate measures were taken to minimise pain or discomfort and animals were handled according to the recommendation of Baumans (2005).

### Homogenate preparation

The basic procedure of Frasco & Guilhermino (2002) was carried out for the homogenisation of the biological matrix. Eighteen fish were collected from the aquarium and sacrificed by decapitation. The head without gills and any vestige of blood was transferred to a test tube containing 1 mL of ice-cold phosphate buffer (0.1 M; pH = 7.2) and homogenised (Ystral Homogeniser mod. D-7982) for 20 s at *ca.*  $4^\circ\text{C}$ . The homogenate was immediately centrifuged at 5,000 g during 3 minutes at  $4^\circ\text{C}$ .

### Enzymatic characterization

The enzymatic characterisation was performed by studying the preferences of substrate of the enzyme present and its behaviour towards selective inhibitors, as described by Garcia *et al.* (2000) but using a different temperature for enzymatic analysis. ChE activity at increasing concentrations of acetylthiocholine (ACT), butyrylthiocholine (BUT) and propionylthiocholine (PRO) (from 0.01 to 2.56 mM) was determined in independent experiments. Eserine sulphate, iso-OMPA and 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) were used as selective inhibitors of ChE, PChE and AChE, respectively. For each inhibitor, enzymatic activity was determined with ACT after an incubation period of 30 min at  $26 \pm 1^\circ\text{C}$  (8  $\mu\text{l}$  of a stock solution prepared in ethanol or ultra-pure water as appropriated was added to 492  $\mu\text{l}$  of *P. reticulata* homogenate). Controls were incubated with 8  $\mu\text{l}$  of ultra-pure water and additional controls with ethanol were also included when appropriate.

### Effects of time and temperature sample preservation

The effects of time and freezing temperature on the activity of wild *P. reticulata* ChE were determined in 48 samples prepared as above described for the characterisation study, using one fish head per sample. In nine samples, defined as the control group, ChE was immediately determined after the preparation of the homogenates. A group of twelve samples was maintained in a freezer ( $-18^\circ$  to  $-20^\circ\text{C}$ ), another group of twelve samples was kept in a deep freezer ( $-48$  to  $-51^\circ\text{C}$ ) and a third group of samples was kept in liquid nitrogen (*ca.*  $-196^\circ\text{C}$ ), during four consecutive weeks. After each week, ChE was determined in four samples of each group.

### ChE and protein determinations

ChE activity was determined by the method described in Ellman *et al.* (1961) adapted to microplate (Guilhermino *et al.*, 1996a), using 0.05 ml of homogenate and 0.250 ml of the reaction solution, at 414 nm, at a temperature of  $26.0 \pm 1^\circ\text{C}$ . The enzymatic activity was determined in quadruplicate and expressed as units (U) per mg of protein (U is 1 nmol of substrate hydrolyzed per minute). Protein concentrations before and after the enzymatic analysis were determined in quadruplicate by the Bradford method (Bradford, 1976) adapted to microplate, using bovine  $\gamma$ -globulin as standard and a Labsystem Multiskan Ascent microplate reader. Protein was standardised to 0.3 mg/ml before ChE determinations.

### Range of ChE activity in wild fish

The normal range of AChE activity of *P. reticulata* head was determined in 39 adult male fish collected in the Capivari Creek in different months of the year.

### Chemicals

ACT, BUT, PRO, DTNB, and bovine  $\gamma$ -globulin were purchased from Sigma-Aldrich, the Bradford of protein assay reagent was bought from BIO-RAD and the remaining substances were acquired from MERCK.

### Data analysis

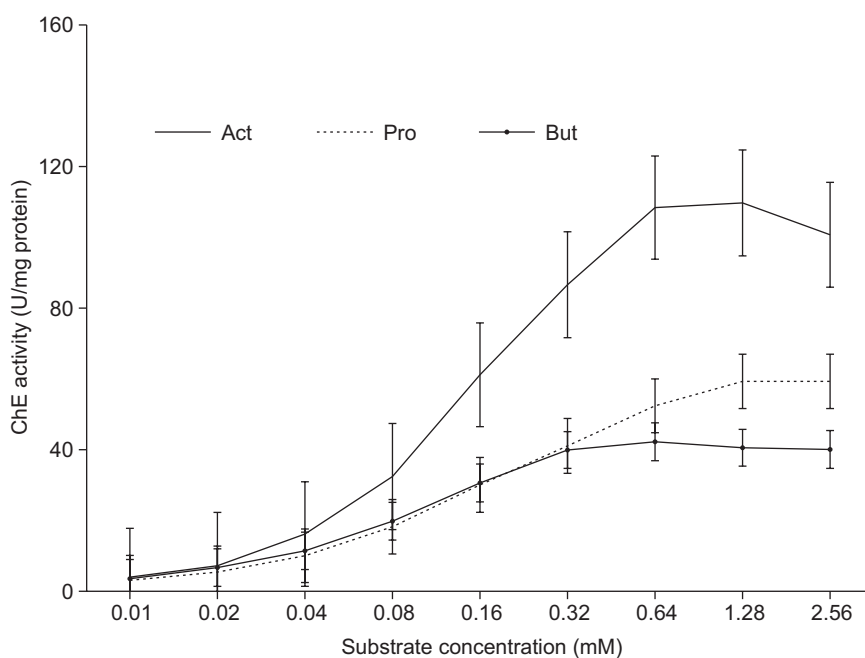
Different treatments in the experiments with selective inhibitors were compared using one-way Analysis of Variance (ANOVA) and the effects of time and temperature of sample

storage were determined using a two-way ANOVA, followed by a Dunnet's test (Zar, 1996). No-observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were determined by the Dunnet multicomparison test.  $IC_{50}$  values were calculated by the Probit Analysis (Finney, 1971). Significant difference was accepted at  $p < 0.05$ .

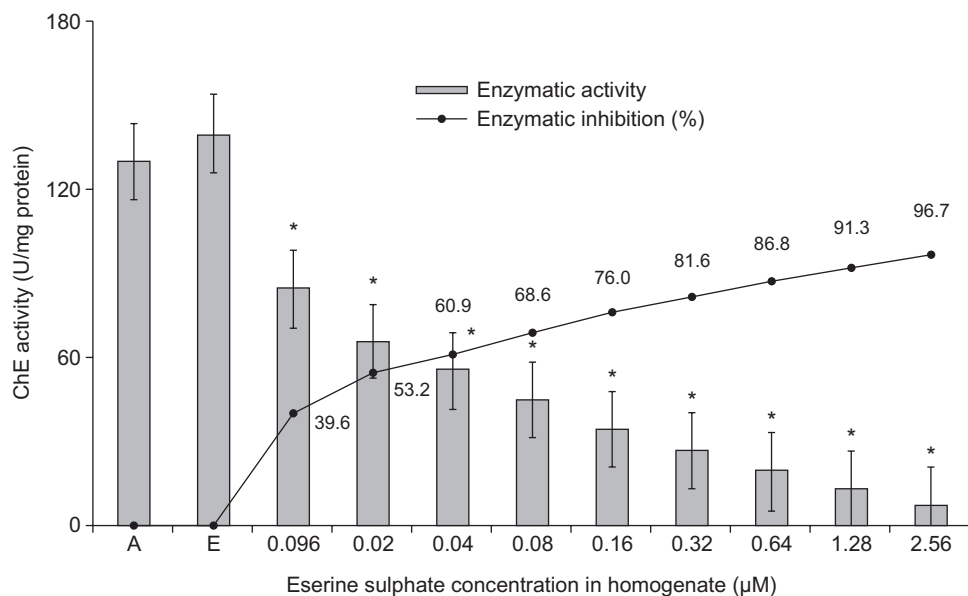
## RESULTS

ChE activity of *P. reticulata*'s head as a function of increasing concentrations of ACT, BUT and PRO is showed in Figure 1. The maximum activity ( $109.68 \pm 3.23$  SE U/mg protein) was obtained with ACT at 1.28 mM and inhibition of ChE activity was observed at the highest ACT concentration tested (2.56 mM). Lower activities were obtained with PRO ( $59.04 \pm 9.5$  SE U/mg protein at 1.28 mM) and BUT ( $40.53 \pm 14.03$  SE U/mg protein at 1.28 mM).

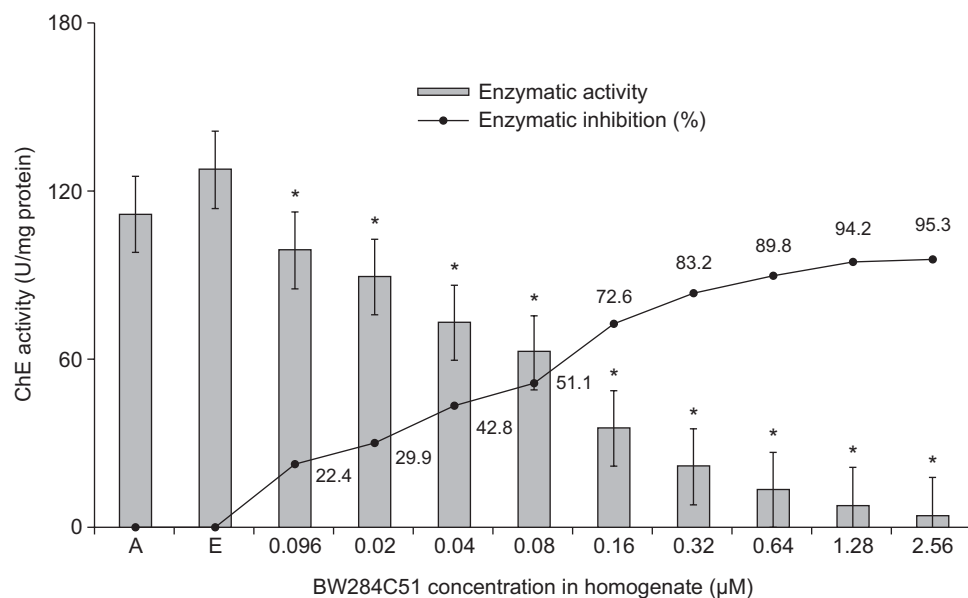
Eserine sulphate ( $F = 100.65$ ;  $p < 0.05$ ; Figure 2) and BW284C51 ( $F = 70.597$ ;  $p < 0.05$ ; Figure 3) significantly inhibited ChE activity. Inhibition was almost complete with  $25.6 \mu\text{M}$  of eserine sulphate (96.7%) and with  $25.6 \mu\text{M}$  of BW284C51 (95.3%) (Figures 2 and 3).  $IC_{50[30\text{min}]}$  for eserine sulphate and BW284C51 were  $0.16 \mu\text{M}$  and  $1.04 \mu\text{M}$ , respectively. LOECs for these compounds were similar ( $0.1 \mu\text{M}$ ) (Table 1). LOECs for these compounds were similar ( $0.1 \mu\text{M}$ ) (Table 1). Soluble ChE from *P. reticulata* head was relatively insensible to iso-OMPA since no significant difference was observed at final concentrations up to  $128 \mu\text{M}$  ( $F = 0.5676$ ;  $p > 0.05$ ; Figure 4).



**Figure 1** – Cholinesterase (ChE) activity in the head of *P. reticulata* as function of acetylthiocholine (Act), butyrylthiocholine (But) and propionylthiocholine (Pro) concentration. Values are the mean of 12 enzymatic determinations per 3 replicate. Bars indicate the corresponding standard error.



**Figure 2** – Effect of eserine sulphate on cholinesterase (ChE) activity in the head of *P. reticulata* (wild organisms). Values are the mean of 12 enzymatic determinations per 3 replicate and corresponding standard error bars. A = distilled water control; E = ethanol control. Asterisk indicates a statistical significance from the control group after one-way ANOVA ( $p < 0.05$ ).



**Figure 3** – Effect of BW284C51 on cholinesterase (ChE) activity in the head of *P. reticulata* (wild organisms). Values are the mean of 12 enzymatic determinations per 3 replicate and corresponding standard error bars. A = distilled water control; E = ethanol control. Asterisk indicates a statistical significance from the control group after one-way ANOVA ( $p < 0.05$ ).

Regarding the effect of storage on *P. reticulata* homogenates, the values of AChE activity measured during four (4) weeks were different statistically and can be identified through the (\*) mark in Figure 5 ( $p < 0.05$ ). During the first week of storage, the value of AChE activity stored in liquid-N was higher ( $155.55 \pm 5.45$  SE U/mg protein;  $F = 6.555$ ;  $p < 0.05$ ) to that kept in freezer  $-20^{\circ}\text{C}$  ( $131.01 \pm 1.36$  SE U/mg protein); however, it did not differ

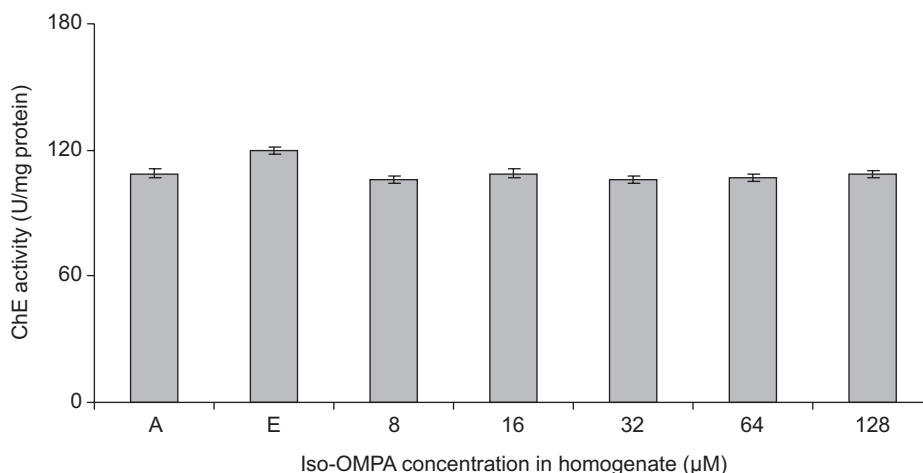
from the enzyme activity measured in controls ( $135.73 \pm 3.81$  SE U/mg protein). Values for the enzymatic activities during four weeks of storage under all temperatures were different from the control and other storage periods.

The range of ChE activity in male fish collected in different periods of the year was  $149.71 \pm 7.72$  SD U/mg protein measure in 156 enzymatic repetitions.

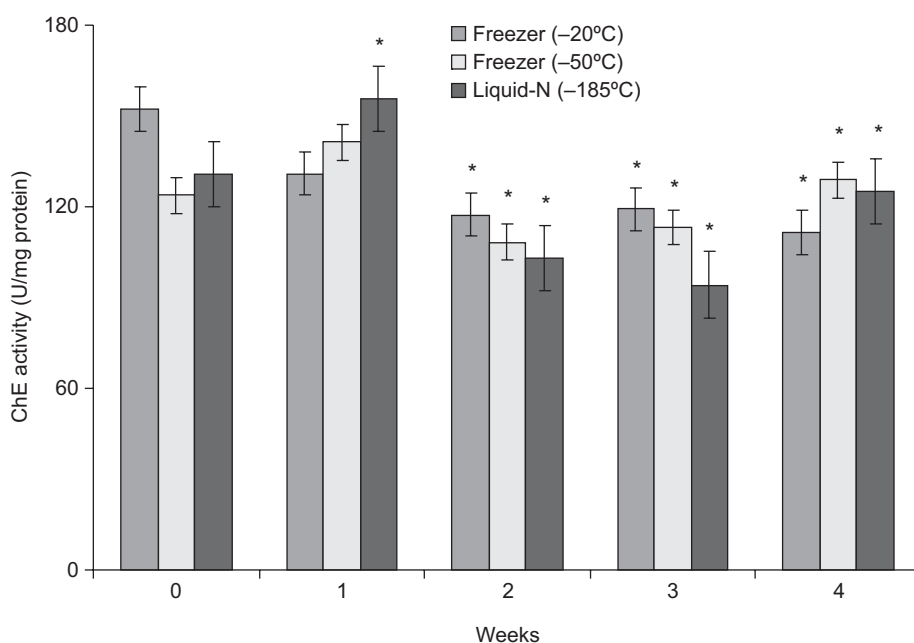
**Table 1** – Effect of specific inhibitors on AChE of *P. reticulata* brain. NOEC – no observed effect concentration, LOEC – lowest observed effect concentration, IC<sub>50</sub> – 50% inhibition concentration, 95% confidence limits are shown within parentheses.

Inhibitor	NOEC (µM)	LOEC (µM)	IC <sub>50</sub> [30 min.] (µM)
Eserine sulphate	<0.1	0.1	0.16 (0.10 - 0.28)
Iso-OMPA	128.0	*	*
BW284C51	<0.1	0.1	1.04 (0.81 - 1.35)

\*No significant inhibition was found up to 128.0 µM.



**Figure 4** – Effect of iso-OMPA on cholinesterase (ChE) activity in the head of *P. reticulata* (wild organisms). Values are the mean of 12 enzymatic determinations per 3 replicate and corresponding standard error bars. A = distilled water control; E = ethanol control.



**Figure 5** – Acetylcholinesterase (AChE) activity in different temperature and period of storage for 4 weeks. Values are the mean of 12 enzymatic determinations per 3 replicate and corresponding standard error bars. Asterisk indicates a statistical significance from the 0 week group after two-way ANOVA ( $p < 0.05$ ), followed by a Dunnet's test.

## DISCUSSION

The almost full inhibition of *P. reticulata* enzymatic activity by eserine sulphate suggests that the activity measured is of ChE and no significant amounts of non-specific esterases were present in the fraction analyzed. The preference of head for ACT over BUTC and PCT, the inhibition by excess of substrate, the high sensitivity to BW284C51 and the relative insensitivity to iso-OMPA indicates that the predominant enzymatic form in the soluble fraction of wild *P. reticulata* head was AChE. This result is in good agreement with the results obtained by Rendón-von Osten *et al.* (unpublished results) which indicated that the main ChE form in head homogenates of commercial *P. reticulata* head was indeed AChE. Moreover, the data provided here is in good agreement with the findings reported in the literature for other species of Teleostei fish (Garcia *et al.*, 2000; Rendón-von Osten *et al.*, 2005; Szegletes *et al.*, 1995; Sturm *et al.*, 2000). Since different populations of the same species may show genetic variability in what concerns ChE forms, the good agreement between the results obtained with commercial and wild fish is particularly important since it enables the comparison of wild populations responses to environmental contaminants with those of commercially available fish for which a considerable amount of data already exist and solves one of the main problems of biomonitoring studies using native populations, that is the lack of suitable reference values (Rendón-von Osten *et al.*, 2006). It also makes ecologically relevant the use of commercial fish in some ecotoxicological studies as substitute of wild fish without the need of extrapolations with ethical advantages.

The sample storage is an important technical procedure frequently used when the number of samples is considerably high. However, some precaution should be taken when using organisms and tissues not used before in the laboratory because some storage conditions may significantly decrease the ChE activity in the samples. In this study, samples stored up to one week at  $-18$  to  $-20$ ,  $-48$  to  $-51^{\circ}\text{C}$ , *ca.*  $-196^{\circ}\text{C}$  did not show significant alterations relatively to ChE activity determined in fresh samples. However, after two weeks of freezing at these temperatures, a significant decrease of ChE activity was observed. This result is in disagreement with the results found for *D. magna* where no significant decrease was observed up to forty days of freezing both at  $-20$  and at  $-70^{\circ}\text{C}$  (Guilhermino *et al.*, 1996b). These evidences may suggest that the loss of activity may depend of the species, tissue or the homogenate preparation (*e.g.* buffer, pH).

The enzymatic activity reduction in homogenates stored at  $-20^{\circ}\text{C}$  for the first week in comparison to samples stored at  $-196^{\circ}\text{C}$  can represent specific alterations of enzymes selected by tissue to compensate changes during the storage of the organ (Cowan & Storey, 2001). These authors indicate that changes in the enzymatic activity *in vitro* can be related with covalent modifications or with proteic reverse phosphorylation. These molecular alterations may induce loss of enzymatic activity of small frozen quantity of enzymes under  $-20^{\circ}\text{C}$  compared to those stored at  $-196^{\circ}\text{C}$ .

As the experiments were independent, the increase from the enzymatic activity observed in samples of *P. reticulata*'s head stored to the  $-196^{\circ}\text{C}$  can be related with the population normal variability (Fossi & Leonzio, 1994).

ChE activity for wild *P. reticulata*'s head obtained in this study ( $149.71 \pm 1.93$  U/mg protein) is higher than the correspondent activity determined in commercial *P. reticulata* head ( $70 \pm 6.82$  U/mg protein) (Rendón-von Osten *et al.*, unpublished results). However, these authors performed ChE determinations at  $25^{\circ}\text{C}$  while a temperature of  $30^{\circ}\text{C}$  was used in this study. Since ChE activity measurements may be affected by temperature (Galgani & Bocquené, 1990), this may contribute to the differences of activity between these two studies. Our results are close to those obtained by Garcia *et al.* (2000), that measured a ChE activity of  $145.1 \pm 44.7$  U/mg protein in *P. reticulata* muscle, and higher than the values reported at lower temperatures for other fish species. For example, an activity of  $7.95$  U/mg protein was found in *Cyprinus carpio* brain (Szegletes *et al.*, 1995), while  $80$ - $90$  U/mg protein were found for *Pomatoschistus microps* head (Monteiro *et al.*, 2006; Vieira *et al.*, 2008) and  $85.5$  U/mg protein were found for *Serranus cabrilla* (Sturm *et al.*, 1999). It should be mentioned, however, that ChE activities may also vary with fish size, as Flammarion *et al.* (2002) observed, with the larger fish having the lower ChE activities.

In conclusion, the results of this study indicate that the predominant form present in the soluble fraction of head homogenates from wild *P. reticulata* collected in the region of Cruz das Almas (Bahia, Brazil) is AChE, the mean activity in the population studied is  $149.71 \pm$  U/mg and the samples may be frozen at  $-20$ ,  $-50$  or  $-196^{\circ}\text{C}$  up to seven days before losing activity. Furthermore, since a good agreement between the results obtained with wild and commercially available *P. reticulata* was obtained, the results of biomonitoring studies and *in situ* tests using ChE as a biomarker may be compared with the responses obtained in laboratorial and/or field tests with commercial fish.

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