Ecotoxicology and Environmental Safety 111 (2015) 236–241

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Circannual rhythms of acetylcholinesterase (AChE) activity in the freshwater fish *Cnesterodon decemmaculatus*

Renata J. Menéndez-Helman [a], Gisele V. Ferreyroa [a], Maria dos Santos Afonso [a,*], Alfredo Salibián [b]

[a] CONICET-INQUIMAE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EHA Ciudad Autónoma de Buenos Aires, Argentina

[b] Programa de Ecofisiología Aplicada (PRODEA), Instituto de Ecología y Desarrollo Sustentable (INEDES), Universidad Nacional de Luján, B6700ZBA Luján, Argentina

ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form
2 October 2014
Accepted 9 October 2014
Available online 31 October 2014

Keywords:
Biomarkers
Acetylcholinesterase
Seasonal variations
Circannual rhythms
Cnesterodon decemmaculatus

ABSTRACT

The use of biomarkers as a tool to assess responses of organisms exposed to pollutants in toxicity bioassays, as well as in aquatic environmental risk assessment protocols, requires the understanding of the natural fluctuation of the particular biomarker. The aim of this study was to characterize the intrinsic variations of acetylcholinesterase (AChE) activity in tissues of a native freshwater teleost fish to be used as biomarker in toxicity tests, taking into account both seasonal influence and fish size.

Specific AChE activity was measured by the method of Ellman et al. (1961) in homogenates of fish anterior section finding a seasonal variability. The highest activity was observed in summer, decreasing significantly below 40% in winter. The annual AChE activity cycle in the anterior section was fitted to a sinusoidal function with a period of 11.2 months. Moreover, an inverse relationship between enzymatic activity and the animal size was established. The results showed that both the fish length and seasonal variability affect AChE activity.

AChE activity in fish posterior section showed a similar trend to that in the anterior section, while seasonal variations of the activity in midsection were observed but differences were not statistically significant.

In addition, no relationship between AChE and total tissue protein was established in the anterior and posterior sections suggesting that the circannual rhythms observed are AChE-specific responses.

Results highlight the importance of considering both the fish size and season variations to reach valid conclusions when AChE activity is employed as neurotoxicity biomarker.

1. Introduction

The pollution of freshwater ecosystems is one of the most critical problems that may affect aquatic environments, since they serve as final receivers of chemically complex mixtures of natural pollutants and xenobiotics. Pollutants (carcinogenicity, hepato-, neuro-, cyto- and geno-toxicity agents, oxidative stress promoters and endocrine disruptors) cause adverse effects on the biota, particularly on fish. These contaminants may cause environmental stress, which often produces abnormal regulatory responses in aquatic organisms (Bradshaw, 2003). In ecotoxicological studies, the concept of *environmental stress* can be defined as a quantifiable alteration of the normal state of a biological system, induced by environmental or anthropogenic factors (Newman and Clements, 2008; Timbrell, 2009). Environmental stress affects different levels of biological organization, ranging from subcellular, individual to population, and may consequently, result in alterations of the ecosystem structure, functionality and integrity (De Coen et al., 2000; van der Oost et al., 2003).

Changes beyond the normal homeostatic limits of a particular parameter can be monitored by selected biomarker measures. The term *biomarker* (WHO, 1993) broadly includes parameters whose alteration reflects the interaction between a particular biological system and the environmental stressors (Conti, 2008; Schlenk et al., 2008). Ecotoxicological biomarkers are frequently used in toxicity bioassays, as well as in environmental quality and risk assessment protocols, as early-warning signals reflecting the adverse sublethal responses to pollutants. Moreover, it is possible to

* Corresponding author. Fax: +54 114 576 3343.
E-mail address: dosantos@qi.fcen.uba.ar (M. dos Santos Afonso).

http://dx.doi.org/10.1016/j.ecoenv.2014.10.017
0147-6513/

quantify the degree of stress from the magnitude of changes in selected biomarkers.

The hydrolase enzymes that are called esterases splits esters into an acid and an alcohol by hydrolysis mediated by water. The enzyme family is constituted by a wide range of diverse enzymes with different substrate specificity, protein structure, and biological function. Acetylcholinesterase (AChE) (EC 3.1.1.7) belongs to this family of enzymes and the physiological role is terminating neurotransmission by hydrolysis of acetylcholine, a neurotransmitter present in the synapses of vertebrates and invertebrates, into acetate and choline. The determination of AChE activity has been used in many ecotoxicological studies (Bradbury et al., 2008). It is a sensitive biomarker for carbamates and organophosphates (OP) pesticides exposure studies (Thompson, 1999; Soreq and Seidman, 2001; van Dyk and Pletschke, 2011). Carbamates provoke the reversible inhibition of AChE, whereas OP induced irreversible inhibition (Thompson, 1999). AChE activity inhibition leads to an accumulation of acetylcholine in the synaptic cleft and thereby to overstimulation of the postsynaptic membrane. These pollutants are known as disruptors of cholinergic nerve transmission, binding the catalytic site of the enzyme and thus preventing the inactivation of acetylcholine. This process on central and peripheral cholinergic receptors, culminate eventually with the development of cardiorespiratory collapse that can lead to death.

Several studies have indicated that besides carbamates and OP, AChE from brain and muscle was also sensitive to other contaminants such as neurotoxic heavy metals, pyrethroids and herbicides (Kumar et al., 2009; Salbego et al., 2010; Richetti et al., 2011).

In the particular case of fish, AChE activity has been shown to be extremely important for many physiological functions such as locomotor capacity, predator evasion, prey location, orientation towards food and feeding, spatial distribution pattern and social interactions (Bradbury et al., 2008). Furthermore, Behra et al. (2002) have reported a non-classical function of the enzyme: AChE was required for neuronal and muscular development in zebrafish embryos (*Danio rerio*). This finding suggests that exposure of fish to toxicants that alter the functionality of AChE during early development could induce alterations in the structure of the fish population.

Cnesterodon decemmaculatus (Jenyns, 1842) (Teleostei, Cyprinidontiformes) is a freshwater non-migratory and live-bearer teleost (Pisces, Poeciliidae), endemic in Neotropical America, which has a wide distribution in a variety of water bodies of the Rio de la Plata basin (Ringuelet et al., 1967; Ringuelet, 1975). *C. decemmaculatus* is one of the native species proposed as suitable species for use in ecotoxicity studies because it is easily handled and acclimatized to laboratory conditions and has been used for *in situ* biomonitoring of aquatic ecosystems (de la Torre et al., 1997, 2002, 2005, 2007; García et al., 1999; Salibián, 2006). Furthermore, AChE inhibition in *C. decemmaculatus* after the exposure to environmental contaminants, such as heavy metals and herbicides, has also been reported (de la Torre et al., 2002; Menéndez-Helman et al., 2012).

In environmental context, important physiological biomarkers may fluctuate temporally and in response to changing periods of organism development (Wendelaar Bonga, 1997). The use of enzymatic activity as a tool to assess responses of organisms exposed to pollutants in toxicity bioassays, as well as in biomonitoring protocols, requires the understanding of the toxic-biological response relationship, the natural fluctuation of a particular biomarker and the validation in the system under study. The aim of this study was to characterize the intrinsic variations of acetylcholinesterase activity in tissues of *C. decemmaculatus* to be used as biomarker in toxicity tests, taking into account both seasonal influence and fish size. This information is relevant for an appropriate use of AChE as biomarker in native freshwater fish.

2. Material and methods

2.1. Animals and experimental design

Fish were captured with hand nets in the Las Flores stream (Latitude 34°29′ S; Longitude 59°07′ W), situated in the headwater of Lujan river basin (NE of the Buenos Aires Province, Argentina, South America), which is recognized as a reference sampling site due to the absence of major sources of anthropogenic pollution (Scarcia et al., 2012). Eight survey samplings at different weather seasons (summer, autumn, winter and spring) were performed over a period of 2 years. In each sampling, 20 adult fish (2–3 cm length) were selected, and a subsample ($n=5$–10) was randomly chosen for analysis. During these 2 years a total of 46 fishes were analyzed ($N=46$).

Semi-static toxicity bioassays were performed following the protocols described for control groups conditions (IRAM, 2007): animals were first acclimatized to glass aquarium conditions (22 ± 2 °C, 12L:12D photoperiod) during 15 days, in dechlorinated tap water, aerated and fed daily *ad libitum* with commercial dried fish food. Then, fish were transferred for a week to a chamber in polypropylene containers with reconstituted moderately hard water (MHW; pH 7.4–7.8; hardness: 80–100 mg L^{-1} $CaCO_3$; alkalinity: 60–70 mg L^{-1} $CaCO_3$) (USEPA, 1993) under the same previous aquarium temperature and photoperiod conditions. MHW was renewed every 48 h, and the ratio of the organisms' weight/MHW volume was kept constant (1 g L^{-1}). After this week, animals were anesthetized in ice-cold chilled water and spinal cord was carefully cut. Each specimen was dissected in three parts: the anterior section (A) corresponded to the head, the midsection (M) included skeletal muscle and viscera and the posterior section (P) contained mainly skeletal muscle. This methodology was adopted considering the small size of the animals, as was previously reported by other authors (Nunes et al., 2005; Varó et al., 2008). Throughout this study, fish pain or discomfort was avoided following the established instructions of animal handling protocols mentioned before (Baumans, 2005).

Homogenates were performed using a glass-Teflon electrically operated tissue homogenizer at 3500–4000 rpm. Homogenization of the anterior section (A) was done in 0.1 M K_2HPO_4 buffer pH 8 as is described in Ellman et al. (1961) for determination of AChE in brain tissue, using a ratio of 1/25 tissue weight/buffer volume. The midsection (M) and the posterior section (P) tissues were homogenized with the buffer typically used for muscle and liver tissue homogenization (phosphate 0.1 M, pH 7.4) (1/20 tissue weight/buffer volume). All homogenates were centrifuged (Hermle Z 216 MK microcentrifuge) at 10,000 g for 15 min at 4 °C; the supernatants were stored at −20 °C and used before 10 days for AChE activity and total tissue proteins biochemical determinations.

Morphometric parameters were determined for seven survey samplings. In this case fish were weighed (W) with an analytical balance, and the total length (L) was measured with a digital caliper, prior to dissection. These values allowed the calculation of Fulton's Condition Factor Index (K) as $K=[(W\text{x}100)/L^3]$. The body weight and length (mean ± SD) of fish ($N=41$) were 132 ± 59 mg and 2.56 ± 0.36 cm, respectively.

2.2. Determination of AChE activity and tissue protein content

The absorbance measurements were carried out in triplicate using a 1 cm path length cuvette and a double beam UV/vis

Pharmasec 1700 Shimadzu Spectrophotometer with UV-Probe Software. Mean value (± SEM) was considered for the analysis.

The activity of acetylcholinesterase (E.C. 3.1.1.7) was determined by the method of Ellman et al. (1961). Each reaction mixture contained: 3 mL of 0.1 M K_2HPO_4 buffer (pH 8), a homogenate aliquot (10 μL for A and M; 20 μL for P), 100 μL of 5,5-dithiobis(2-nitrobenzoic acid) solution (10 mM DTNB) and 100 μL of substrate (0.075 M acetylthiocholine iodide). The hydrolysis reaction rate of substrate mediated by AChE was followed spectrophotometrically by absorbance measurements at 412 nm for 2 min, at 8-s intervals. In each case, the absorbance was corrected by subtracting the background for a reagent blank (without sample). AChE activities were calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1}.

AChE activity was expressed as specific activity units (1 U = 1 nmol of substrate hydrolyzed min^{-1} mg $protein^{-1}$).

Total protein content was quantified by Lowry's method. The absorbance was measured at 650 nm, using Bovine Serum Albumin (BSA) as standard in the range 0–30 μg. The total protein content was expressed as mg g wet $tissue^{-1}$.

2.3. Statistical analysis

The temporal comparison of AChE activity and total protein content were performed using Bonferroni´s test for multiple comparisons (GraphPad Prism Software). The value of $p < 0.05$ was considered to indicate a significant difference in all statistical analysis. A non-linear regression (Waveform, Sine, 4 Parameter; SigmaPlot Software) was performed using the following sinusoidal function:

$$f(x) = y = y_0 + A\sin((2\pi x / b) + c) \quad (1)$$

where $f(x)$ represents AChE activity, x is the variable time expressed in months, the equation parameters A and b are the amplitude and period, respectively.

AChE and fish length dependence were analyzed using a linear regression. Also, two-way ANOVA using Bonferroni post test was performed to compared simultaneously the seasonal influence (winter *vs.* spring–autumn) onto AChE and fish length.

Furthermore, AChE, fish length and seasonal dependence were analyzed using a multiple regression (3D, quadratic surface; SigmaPlot Software) performed by the following quadratic surface function:

$$f(x, y) = z = z_0 + ax + by + cx^2 + dy^2 + exy \quad (2)$$

where $f(x)$ represents AChE activity and x and y are fish length and time, expressed as season, respectively.

2.4. Chemicals

All reagents were of analytical grade and solutions were prepared using milli-Q water. BSA, DTNB and acetylthiocholine iodide were purchased from Sigma (St. Louis, MO, USA).

3. Results and discussion

The basal levels of physiological biomarkers are a relevant tool to be used in toxicity tests, as well as in biomonitoring protocols for fish species as sentinel organisms (Schlenk et al., 2008). In this sense, AChE activity in *C. decemmaculatus* captured in the Las Flores stream at different weather seasons over a period of 2 years was evaluated.

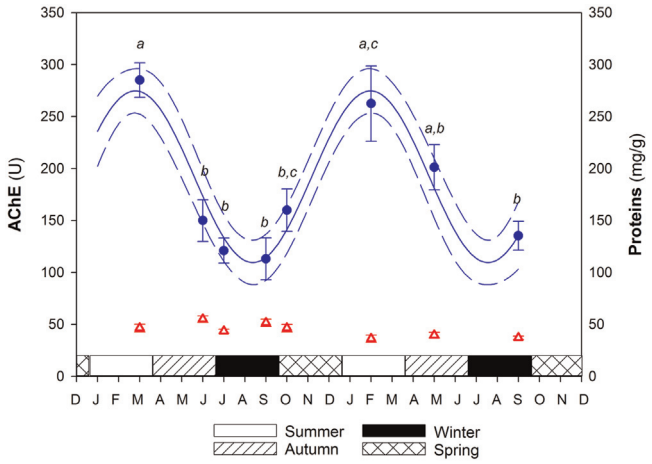

Fig. 1. : Acetylcholinesterase activity (AChE, filled circles) and total tissue proteins (unfilled triangles) in homogenates of the anterior section of *Cnesterodon decemmaculatus* captured during different seasons of the year. Values were expressed as means ± SEM for each group of fish. The curve was fitted using a sinusoidal function (solid line), the 95% confidence band is also plotted (dashed lines). Significantly different values are indicated with distinct letters (a, b, c).

3.1. AChE activity in the anterior section of C. decemmaculatus

The AChE activity values from the anterior section were plotted *vs.* time and a seasonal cyclic variability was established, showing a waveform pattern (Fig. 1).

The greatest AChE activity was observed in summer (around 285 U, beginning March), reaching a minimum value, below 40% (around 113 U, beginning September) in winter, while intermediate experimental values were recorded in autumn and spring. Therefore, AChE activity values in summer were two fold greater than winter values. Moreover, the AChE activity values corresponding to summer and winter were found to be significantly different ($p < 0.01$) by Bonferroni's test statistical multiple comparisons when all seasons (summer, autumn, winter and spring) were simultaneously compared.

The obtained sinusoidal function (Eq. (1)) was

$$f(x) = 192.2 + 82.6\sin((2\pi x/11.2) + 6.3)$$

The above equation shows the theoretical minimum value ($y_0 - A$) of 109.6 U in August and the maximum value ($y_0 + A$) of 274.8 U in February. The coefficient b was 11.2 months, confirming an annual cycle of enzyme activity. The determination coefficients and statistical p-level obtained were $R^2 = 0.9421$ and $p < 0.01$, respectively, indicating that the sinusoidal function is adequate to

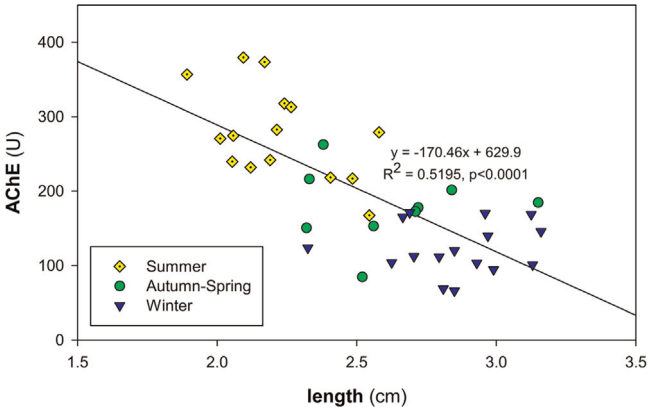

Fig. 2. : AChE activity of the anterior section *vs.* length of *Cnesterodon decemmaculatus*.

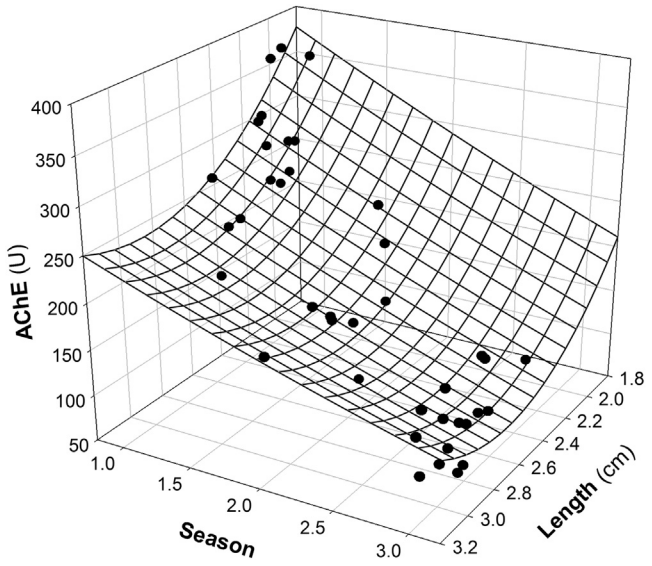

Fig. 3. : Multiple regression plot of *Cnesterodon decemmaculatus* anterior section AChE activity as a function of fish length and season. The season was converted to a numerical variable: 1: summer; 2: spring–autumn and 3: winter.

represent the temporal behavior of AChE in the anterior section of *C. decemmaculatus*.

Moreover, it is noteworthy that seasonal variations in tissue proteins were not observed in the anterior section (Fig. 1), the overall mean value being 44 ± 1 mg g^{-1} (mean $\pm$ SEM, $N=46$). The relation between AChE activity and tissue proteins was analyzed (data not shown), the linear regression showed a low determination coefficients ($R^2=0.007$) indicating that there was no relation between these parameters. This result suggests that the seasonal variability in AChE activity is not a non-specific response because an effect on AChE activity was determined while no effect on the tissue protein content was observed.

Temperature and photoperiods are the most important exogenous factors that regulate annual rhythms in fish (Thomas, 2008). These two factors were kept constant before the biochemical determinations. In fact, the experimental data showed that fish acclimation (typically performed in toxicity bioassays) did not reverse seasonal rhythms.

In relation to the seasonal pattern determined in *C. decemmaculatus*, a few reports have also described natural variations concerning AChE activity in fish. Chuiko et al. (1997) determined the existence of seasonal fluctuations of brain AChE activity in freshwater fish *Rutilus rutilus*, showing the highest enzyme activity values at the beginning of summer and the lowest in winter. Moreover, in the migratory species *Anguilla anguilla* significant differences in the AChE activity among seasons were also reported, with the highest activities found in summer and the lowest in autumn (Guimaraes et al., 2009).

Furthermore, the fish Condition Factor, which is an indicator of its well-being, showed similar values over the analyzed period. The *K* value was 0.74 ± 0.02 (mean $\pm$ SEM, $N=41$) without significant differences throughout the year. However, the size of the fish was not homogeneous throughout the different seasons; when AChE activity was plotted against the fish length, a relationship between these parameters was evident (Fig. 2), where AChE activity decrease with increasing fish size.

Beauvais et al. (2002) previously reported that some parameters such as euthanasia method or sex of fish do not affect the brain enzyme activity for bluegill (*Lepomis macrochirus*). These authors also point out that the size was the only variable that requires being controlled. Similar conclusions were also reported for fish muscle AChE activity (Flammarion et al., 2002), for fish brain AChE activity (Chandrasekara and Pathiratne, 2007), and also for crustacean amphipods (Xuereb et al., 2009); in all cases the largest size have the lowest enzyme activities being these results similar to those presented in this study (Fig. 2).

A seasonal variation in size could be the reason for the observed seasonal variation in AChE. However, the fish lengths were 2.33–3.16 cm (2.85 ± 0.05) and 2.32–3.15 cm (2.61 ± 0.09) and AChE activity values 66–171 U (123 ± 9) and 85–263 U (178 ± 16) for winter and autumn–spring, respectively. In this case, fish length and AChE activity were expressed as the mean $\pm$ SEM of all individual values for each season. It should be noted that fish length in winter was similar to that in autumn–spring while AChE activity in winter was lower than in autumn–spring (Fig. 2).

Two-way ANOVA using Bonferroni post test comparing simultaneously the seasonal influence (winter *vs.* spring–autumn) onto fish length and AChE was made. Results showed no significant differences ($p > 0.05$) for the fish length while for AChE activity was significantly different ($p < 0.001$). Thus, the fish length would not be the only factor affecting AChE activity variations.

In order to further explore different factors that could explain this behavior, a multiple regression analysis was conducted. The *xyz* graph is shown in Fig. 3, where *x* is the length, *y* the season and *z* the anterior section enzyme activity.

AChE activity was highest in the smaller sized fish during the summer and lowest in the larger size fish during the winter. The experimental values were fitted using a quadratic surface function (Eq. (2)), and fitting parameters for the surface response regression are listed in Table 1. The determination coefficient and significant *p*-level were $R^2=0.7306$ and $p < 0.0001$, respectively, indicating a good agreement between the fitted curve and the experimental results confirming that AChE activity is a function of fish length and season.

The relative weight of each variable on AChE activity was estimated using the quadratic surface function for four combinations

Table 1
Quadratic function parameters for surface regression. AChE activity estimated using the quadratic surface function for four combinations of length and season. Theoretical length values were chosen covering the experimental fish length range.

	Quadratic surface regression						
	Season (y)	Length (x)	$f(x,y)=$	z_0 *1360.97*	$ax+cx^2$ *−739.29x+127.05x²*	$by+dy^2$ *−111.70y+3.38y²*	$+exy$ *+15.92xy*
AChE estimation	1	1.9	**337**	1361	−946	−108	30
	1	3.2	**239**	1361	−1065	−108	51
	3	1.9	**201**	1361	−946	−305	91
	3	3.2	**144**	1361	−1065	−305	153

Summer=1; Winter=3.

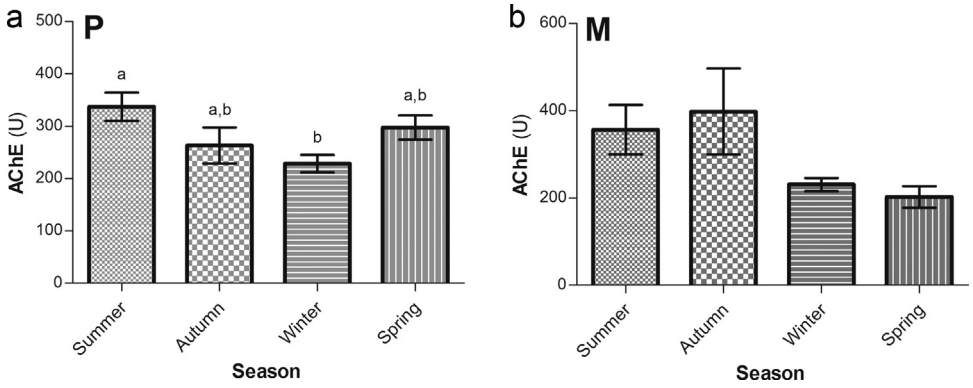

Fig. 4. : AChE activity in the posterior (P) and midsection (M) of *Cnesterodon decemmaculatus* at different seasons. Values expressed as means ± SEM for each group. Significantly different values are indicated with distinct letters (a, b).

of length and season: minimum–maximum, maximum–minimum, maximum–maximum and minimum–minimum (Table 1).

These results suggest that both the length and season are relevant parameters to determine AChE activity rhythms in fish anterior section (Fig. 3 and Table 1).

AChE activity inhibition above 20% has been suggested as indicative of exposure to anticholinesterase agents (Ludke et al., 1975). However, the experimental activity data for *C. decemmaculatus* anterior section determined in this work showed variations of over 20%, even though fish were not previously exposed to any inhibitory agent. It is important to note that these season AChE variations can not be reversed by fish acclimation. Therefore, the natural intrinsic variations in control fish should be taken into account when conducting toxicity tests and biomonitoring programs, using AChE as a biomarker. The response to stress factor can not be assumed to be the same under all conditions, since the sensitivity of the enzyme to the stressor may diverge due to the existence of circannual rhythms. This fact reasserts the importance of considering seasonality when performing replicates of bioassays. In this sense, a rigorous study of the toxic–response relation is also required for different conditions to analyze a particular contaminant.

It is worth mentioning that AChE decrease is not necessarily fatal. In some species, fish survival has been observed even in brain AChE activity inhibition of 90–95% (Sturm et al., 2000; Fulton and Key, 2001; Ferrari et al., 2004). Moreover, several reports have indicated that AChE inhibition by herbicides and insecticides may be reversible (Dembélé et al., 1999; Cattaneo et al., 2011), suggesting *de novo* protein synthesis or molecular rearrangements in the enzyme structure.

3.2. AChE activity in the posterior and midsection

Vertebrate cholinesterases (ChE) have been classified into two main groups, depending on substrate hydrolysis and sensitivity to inhibitors: Acetyl ChE (AChE), which preferentially hydrolyzes acetyl esters such as acetylcholine; and butyryl ChE (BChE), which preferentially acts on butyrylcholine. The brain of most teleost fish contains mainly AChE (Stringuetti et al., 2008), while body muscle may have either AChE or a mixture of AChE and BChE. In this sense, Varó et al. (2003) found esterase inhibition differences between head and muscle of the European sea bass. Considering these facts, AChE activity in the posterior (P) and midsection (M) of *C. decemmaculatus* was determined for comparative analysis (Fig. 4).

AChE activity seasonal changes at the P section exhibited a comparable profile to that of the anterior section, where the greatest activity corresponded to summer, while a lowest activity with a significant decrease, below 46%, occurred during winter months ($p < 0.05$). Total tissue protein of posterior section was 71 ± 4 mg g^{-1} (mean ± SEM, $N = 34$). The relation between AChE activity and tissue proteins was analyzed, but the linear regression, as well as for anterior section, was not a good estimation with a determination coefficient of $R^2 = 0.0956$. Also in this case, there was not relation between AChE activity and tissue proteins suggesting that the seasonal variability in enzyme activity is not a non-specific response.

Another tendency was observed in the M section, where the maximum and minimum AChE activity was recorded in autumn and spring, respectively, although the variations were not statistically significant. Even more, the total tissue proteins content (107 ± 6 mg g^{-1}, $N = 34$) and AChE showed an inverse linear regression that was statistically significant ($p < 0.01$) although with a low coefficient of determination ($R^2 = 0.2034$), suggesting that in this case AChE variability is non-specific.

4. Conclusions

Environmental stress may cause adverse effects on different levels of local biota organization and disturb the structure, functionality and balance of a particular ecosystem. The magnitude and type of disturbances depend on various factors: physicochemical parameters (concentration, chemical profile of the media, temperature, photoperiod, duration of exposure, etc.) or biological parameters of the test organisms (species, age, sex, nutritional status, diet, genetics, physiology, reproduction, etc.).

The use of biomarkers in risk assessment studies and toxicity bioassays requires the identification and measurement of additional parameters that may affect their biochemical responses. In this regard, it is necessary to have precise knowledge of biomarker basal levels and their variations. This relevant information is required to effectively differentiate test species contaminant-induced effects from their natural biological cycles.

The main contribution of this work is to highlight the importance of considering both the fish size and the circannual rhythmic variations of enzyme activity, in toxicity bioassays and biomonitoring programs, to reach valid conclusions when AChE activity is used as neurotoxicity biomarker.

Acknowledgements

The authors acknowledge the Universidad de Buenos Aires (UBA, UBACyT 20020100100750 grant), the Departamento de Ciencias Básicas, Universidad Nacional de Luján (UNLu), Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) and Ministerio de Ciencia, Tecnología e

Innovación Productiva, Agencia Nacional de Promoción Científica y Tecnológica, Fondo para la Investigación Científica y Tecnológica (MINCyT–ANPCyT–FONCyT, PICT 2007-00371 grant) for financial support. Authors are also thankful to Rubén H. Lombardo for his suggestions for the statistical analyzes.

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