Acetylcholinesterase activity, antioxidant defenses, and lipid peroxidation in the clam *Semele solida*: Can this species be used as a bioindicator?

Actividad acetilcolinesterasa, defensas antioxidativas y peroxidación lipídica en el molusco bivalvo *Semele solida*: esta especie ¿Podría ser utilizada como bioindicador?

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Resumen.- Se evalúa en terreno un conjunto de parámetros bioquímicos: actividad acetilcolinesterasa (AChE), glutation-S-transferasa (GST), concentración de glutation (GSH) y peroxidación lipídica, en el molusco bivalvo *Semele solida,* con el objeto de analizar su relación con el efecto del estrés ambiental generada por la actividad antrópica, en tres estuarios del Pacífico Sureste: estuario Coliumo (Bahía Coliumo), estuario Lenga (Bahía San Vicente) y estuario Andalién (Bahía Concepción). Coliumo es el estuario con menor estrés ambiental. Para ello, se seleccionó 30 individuos juveniles de *S. solida* desde cada sitio. Se analizó en el homogenizado de branquias o tejido digestivo de cada individuo: actividad AChE; actividad GST, concentración GSH, concentración de malonildialdehído (peroxidación lipídica) y contenido de proteínas. Los parámetros bioquímicos analizados en los organismos recolectados en el estuario Coliumo difirieron significativamente de los recolectados en el estuario Andalién, los que presentaron menor actividad AChE en tejido branquial (2189,9 ± 189,6 µmol min⁻¹mg proteína⁻¹) y concentración intracelular de GSH (59,8 ± 13,3 µM) y en glándula digestiva mayor actividad GST (614,9 ± 92,3 µmol min⁻¹mg proteína⁻¹) y mayor grado de peroxidación lipídica (31,9 ± 7,4 nmol MDA mL⁻¹). Se observó una estrecha relación entre la respuesta de los parámetros bioquímicos analizados en *S. solida* y el nivel de estres ambiental presente en el área. Dado la sensibilidad de *S. solida* respecto de los parámetros analizados, se recomienda utilizar a esta especie como bioindicador en programas de vigilancia ambiental en la zona costera del Pacífico sureste.

Palabras clave: Bioindicadores, biomarcadores, bivalvos marinos, programas de vigilancia ambiental

Abstract.- We analyzed the relationships between biochemical parameters: acetylcholinesterase activity (AChE); glutathione S-transferase activity (GST); glutathione concentration (GSH) and lipid peroxidation; in the bivalve mollusc *Semele solida,* with the effects of environmental stress (anthropogenic activity), from three estuaries in Eastern South Pacific bays: Coliumo estuary (in Coliumo Bay), Lenga estuary (in San Vicente Bay) and Andalién estuary (in Concepción Bay). Coliumo is the estuary with minor environmental stress. Thirty juveniles from each site were selected for individual analyses in homogenized of gill or digestive gland tissue to assess AChE activity, GST activity, GSH concentration, malonyldialdehyde concentration (MDA) (lipid peroxidation), and protein content. The biochemical parameters analyzed in specimens from Coliumo estuary, differed significantly from that in Andalién estuary, with lower AChE activity in gill tissue (2189.9 ± 189.6 µmol min⁻¹mg protein⁻¹) and intracellular GSH levels (59.8 ± 13.3 µM), and the highest lipid peroxidation (31.9 ± 7.4 nmol MDA mL⁻¹) and GST activity (614.9 ± 92.3 µmol min⁻¹mg protein⁻¹). The biochemical parameters in *S. solida* were closely related to the sector's stress. Due to this bivalve sensitivity, is recommended as bioindicator for use in programs of environmental alertness in the Eastern South Pacific coastal zone.

Key words: Biomarkers, bioindicadores, marine bivalves, programs of environmental alertness

INTRODUCTION

The exposure/effect of some contaminants can be monitored at various levels, in sensitive's endemic species (functioning as bioindicators), for the assessment of the quality of the coastal environment (Funes *et al.* 2006). Bioindicators are sentinel organisms used to assess the exposure and biological effects of pollutants in the ecosystems in that they reside. Within bioindicators, a variety of molecular parameters respond to environmental stress and are used as early-warning signals -or biomarkers- that alert before the appearance of irreversible damages

to the ecosystem (Bonilla-Valverde *et al.* 2004, Sheehan & McDonagh 2008). Biomarkers can be defined as "the measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response" (Bodin *et al.* 2004, Sarkar *et al.* 2006). For example, acetylcholinesterase activity (AChE) is inhibited when organisms are exposed to organophosphate and carbamate pesticides (Vioque-Fernández *et al.* 2007, Sarkar *et al.* 2006). Other analysis can be done on antioxidant defences such as glutathione S-transferase and glutathione, overproduction of oxygen-reactive substances and oxidative stress (Boelsterli 2003).

There are two enzyme activities used as biomarkers: Glutathione S-transferase (GST) and Glutathione (GSH). The GST is an antioxidant enzyme found in the cytosol. It acts in phase II reactions, inactivating electrophilic substances (those that attract electrons) by conjugation (Cheung *et al.* 2002). GSH is a tripeptide thiol composed of glycine, cysteine, and glutamic acid found in large concentrations in most cells, plays an important role in protecting these from oxidative damage. GSH acts as a co-factor in phase II biotransformation reactions carried out by GST (López-Barea & Gómez-Ariza 2006) cleans cells of free radicals and reactive oxygen species, and detoxifies hydrogen peroxides and lipid hydroperoxides through reactions catalyzed by glutathione peroxidase (Ahmed 2005).

Other usefull process, as biomarker, is lipid peroxidation that occurs in plants and animals. It consists of the destruction of the lipid membrane and the production of peroxides and other sub-products such as aldehydes. Malonyldialdehyde (MDA), formed by the rupture of unsaturated fats, is used as an index for lipid peroxidation. Anthropogenic contaminants such as metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and residues from pesticides and the cellulose industry induce antioxidant and lipid peroxidation defences (Van der Oost *et al.* 2003, Sheehan & McDonagh 2008).

Monitoring programs have shown that bivalve molluscs (informally including mussels, clams, oysters and scallops) are good indicators of environmental stress, they have ample geographic distribution, have adapted their gills as filter-feeding organs, often have a well-developed byssus apparatus allowing them to attach to rocky substrates and they have shown considerable resilience and now occupy niches in a wide range of aquatic environments (Goldberg & Bertine 2000, Vidal *et al.* 2002, Sheehan & McDonagh 2008).

The objective of this study was to analyse AChE activity, antioxidant defences, and lipid peroxidation in the bivalve *Semele solida* as bioindicators of environmental stress. This bivalve mollusc is commercially important species has a wide latitudinal distribution in the Eastern South Pacific, from Callao, Peru, to the Magallanes, Chile.

We analysed and compared the behaviour of these biomarkers in organisms collected from estuaries in three bays of the Eastern South Pacific with different degrees of alteration: i) Lenga estuary in San Vicente Bay, ii) Andalién estuary in Concepción Bay, and iii) Coliumo estuary in Coliumo Bay. The latter was chosen as a reference site due to its low intervention (very low anthropogenic activity). This study responds to the need to identify native species off the coast of the eastern South Pacific that can be used in environmental monitoring programs.

MATERIAL AND METHODS

STUDY AREA

The study area is located in the Eastern South Pacific (central Chile), where the temperature ranges from 9 to 13.5ºC throughout the year and the difference in the normal tide height does not exceed 0.8 m (Ahumada *et al.* 1983). The surface of San Vicente Bay is 13.2 km, with an industrial complex including fish-processing, steel, petrochemical, and associated industries on its north shore (Ahumada *et al.* 1989). In the south-eastern sector of the bay, Lenga estuary (*ca.* 3.2 km^2). Here, counter-clockwise circulation introduce water from the continental shelf adjacent to the southern and moves water toward the northeast, with a water residence time estimated to be about 20 hours (Ahumada *et al.* 1989).

The surface of Concepción Bay is 190 km^2. At present, several uses of the coastal zone make steady contributions of contaminants to the bay: a) port activity at Talcahuano to the west and Lirquén, Penco, and Tomé to the east; b) oil unloading terminals at the bay mouth; c) industrial and artisanal fishing; d) an effluent of waste water without treatment discharge at the bay head; and e) navy shipyards to the west. The Andalién River flows into the south-eastern sector at the head of the bay. Along its trajectory, this river receives sewage without treatment from nearby towns and residual chemical substances from agricultural and forestry activity in the coastal ranges (Rudolph *et al.* 2002, Fuentes-Ríos *et al.* 2005, Altamirano-Chovar *et al.* 2006). Although south and south-westerly winds are responsible for summer upwelling, the water column is

deficient in oxygen; the water has a mean residence of three days (Ahumada *et al.* 1983).

Coliumo Bay is located to the north of the bay system (Table 1) and the Coliumo estuary flows into the head of the bay. This area is a tourist center in summer (*i.e.*, 2-3 months) and during the rest of the year, has a low population (3,057 inhabitants, Censo 2002) and daily water flushing. Given its low levels of alteration, the area is used herein as a reference area (Rudolph *et al.* 2008).

BIOLOGICAL MATERIAL

In October, during the summer of 2006, 70 juvenile *S. solida* (5.5 ± 1 cm in length) were collected in each study area, Andalién estuary (from Concepción Bay); Lenga estuary (from San Vicente Bay) and Coliumo estuary (from Coliumo Bay) (Table 1). The individuals were taken from each sector to the laboratory in buckets with water, where they were kept for 24 h (12 ± 1ºC; 20 ± 4 psu; 8.1 ± 1.5 mg L^{-1} dissolved oxygen; pH 8.2 ± 0.2; 12:12 photoperiod).

BIOCHEMICAL ANALYSIS

For the biochemical analysis, 30 males were selected from each sector and analysed individually. Gill and digestive gland tissue were homogenised in a potassium phosphate buffer 50 mM, KCL 0.1 mM, EDTA 0.1 mM, pH 7.4, and 20 % glycerol. The homogenised tissue was centrifuged at 12,000 x g at 4ºC for 30 min and the supernatant was used for the analysis. The AChE activity was determined according to Ellman *et al.* (1961), GST activity according to Jakoby (1985), and GSH concentrations according to Beutler (1975). The degree of lipid peroxidation quantified by analysing substances reactive to thiobarbituric acid and determined as the concentration of malonyldialdehyde (Buege & Aust 1978). The protein concentration measured according to Bradford (1976) using bovine albumin as a standard solution.

STATISTICAL ANALYSIS

For the analysis of normality, the Shapiro-Wilk test was applied and the Cochrane test was used for the homogeneity of the variance. The statistical analysis of the data was carried out with statistical software (STATISTICA version 6.0 2001); Pearson's correlation analysis, one-way analysis of variance (ANOVA) and Tukey's *post hoc* test, were performed. The results were determined to be significant at $P < 0.05$.

RESULTS

The highest AChE activity measured in the gill tissue was observed in bivalves from the Coliumo estuary (3610.1 ± 20.8 µmol min⁻¹mg protein⁻¹). The lowest AChE activity was found in the individuals inhabiting the Andalién estuary (Table 2); this activity was significantly lower than in the other study areas ($P < 0.001$; $F_{(2, 87)} = 6.8$).

Table 1. Geographic location of studied areas in the South Pacific, central Chile / Localización geográfica de las áreas muestreadas en la costa Pacífico sur, Chile central

Study areas	Bays	Localization
Coliumo Estuary	Coliumo Bay	36º32.5'S 72º57'W
Andalién Estuary	Concepción Bay	36º44'S 73º0.05'W
Lenga Estuary	San Vicente Bay	36º45.7'S 73º10.3'W

Table 2. Average ± standard deviation among estuaries of biochemical parameters analyzed in *Semele solida* (n = 30) for each estuary. * = indicated a significant differences (*P < 0.05*) / Promedio ± desviación estandar entre estuarios de los parámetros bioquímicos analizados en *Semele solida* (n = 30) de cada estuario. * = indica diferencias significativas (*P < 0.05*)

	AChE activity (µmolmin⁻¹mg prot⁻¹)	GST activity (µmolmin⁻¹mg prot⁻¹)	GSH (µM)	Lipid peroxidation (nmol MDA ml⁻¹)
Coliumo Estuary	3610.1 ± 202.8	139.4 ± 48.2 *	89.40 ± 19.46	12.9 ± 7.9
Andalién Estuary	2189.9 ± 189.6 *	614.9 ± 92.3	59.80 ± 13.30 *	31.9 ± 7.4 *
Lenga Estuary	3046.6 ± 203.2	507.7 ± 98.6	69.84 ± 6.29 *	17.7 ± 8.2

The GST activity measured in the digestive gland of *S. solida* (139.4 ± 48.2 μmol min⁻¹mg protein⁻¹) in the individuals from the Coliumo estuary was significantly lower ($P = 0.0001$; $F_{(2, 87)} = 15.6$) than that of the individuals from the Lenga and Andalién estuaries (Table 2).

The average intracellular GSH concentration measured in the digestive gland of the organisms collected in the Coliumo estuary had the highest average value (89.4 ± 19.5 μM), followed by the individuals from the Lenga and Andalién estuaries (Table 2). The statistics revealed significantly different GSH concentrations in the digestive glands of organisms from the three study areas ($P = 0.0001$, $F_{(2, 87)} = 33.9$).

The degree of lipid peroxidation measured as the average intracellular MDA concentration from the three study areas indicated significant differences ($P = 0.0001$; $F_{(2, 87)} = 15.3$). The MDA concentration was significantly higher in the individuals from the Andalién estuary (31.9 ± 7.4 nmol ml⁻¹) than in those from Lenga and Coliumo (Table 2).

The degree of association between the studied bioindicators was high, *i.e.,* $r = -0.91$ and $P < 0.05$ for specific enzymatic GST activity (expressed as μmol min⁻¹mg protein⁻¹) *vs.* intracellular GSH concentration (μM); and $r = -0.88$ and $P < 0.05$ for the GSH concentration (μM) *vs.* MDA concentration (nmol ml⁻¹).

DISCUSSION

Three areas of high ecological and social value were chosen for studying the response of *S. solida* to ambiental stress; we sought to identify correlations between sector's alteration and the biochemical responses of this filtering bivalve. Given the low intervention in the Coliumo estuary the magnitude of the biochemical parameters (biomarkers) analyzed was the awaited one *i.e.,* high AChE activity and low lipid peroxidation.

In San Vicente Bay, due to the circulation, the waste from the industrial complex in the northeast does not significantly affect the subtidal Lenga estuary in the Southeast, which is inhabited by *S. solida*. Waters from the continental shelf enter through the south and have low residence times within the bay (Ahumada *et al.* 1989). This would explain the observed -AChE activity and the degree of lipid peroxidation- did not differ with respect to the individuals from Coliumo estuary.

The individuals from the Andalién Estuary (Concepción Bay) showed higher levels of biochemical alteration in the tissues (*i.e.,* low AChE activity, more GST activity, a lower GSH intra-cellular concentration and a higher degree of lipid peroxidation). These resutls concur with other studies reported in this area, as consequences of port activity, terminals for discharging petroleum, industrial and small-scale fishing, shipyards and the inflow of sewage (Rudolph & Rudolph 1999). This is coincidental with information observed in other organisms in the same place: *e.g.,* high benzo(a)pyrene hydroxylase activity in *P. microps* and *C. coronatus* (Rudolph & Rudolph 1999), high EROD activity in *Schroederichthys chilensis* (Fuentes-Ríos *et al.* 2005), AChE inhibition in *S. solida* (Srain & Rudolph 2008).

The inhibition of AChE activity observed in the organisms collected in the Andalién estuary sector could be attributed to the presence of some organophosphate-type compound anticholinesterase chemical used for forestry plantations and/or agricultural activities in the coastal zone. Although organophosphate pesticides have short average life spans, they could be transported to the Andalién River estuary where they would mix with residues in untreated municipal waters from surrounding populations (~10,000 inhabitants), converging in the estuary through tidal action. This would coincide with observations in similar conditions of the Black Sea made by Baršienė *et al.* (2006) and Kopecka *et al.* (2006).

Likewise, increased GST activity in the organisms would indicate the presence of xenobiotics (*i.e.*, agricultural and forest activity and maritime traffic) that generate stress in the cellular metabolism of the organisms inhabiting the Andalién and Lenga estuaries. GST is an antioxidant enzyme found in cytosol that acts in biotransformation reactions of phase II xenobiotics. Its function is to combine, *i.e.*, to inactivate electron deficient electrophilic compounds (many of which are carcinogenic). These reactions are vitally important in detoxifying xenobiotics and contaminants of an anthropogenic origin (Cheung *et al.* 2002).

Increased GST activities have been reported also in organisms exposed to high organochlorate concentrations and polychlorinated biphenyls (Cheung *et al.* 2002). Gownland *et al.* (2002) reported the induction of GST activity in populations of *M. edulis* exposed to polycyclic aromatic. Moreira & Guilhermino (2005) observed high GST activity in molluscs inhabiting an area close to an industrial center with intense maritime traffic.

The intracellular GSH concentration was lower and the degree of lipid peroxidation higher in the organisms

from the Andalién estuary. GSH detoxifies hydrogen peroxides and lipid hydroperoxides through reactions catalyzed by glutathione peroxidase (Ahmed 2005), and acts as a cofactor in the phase II transformation reactions carried out by GST (Jakoby 1985). The decreased GSH concentration can be explained by the high levels of free radicals that would be reduced by the GSH, and by lower activity of glutathione reductase, the enzyme responsible for regenerating GSH through a reaction dependent on Nicotinamide adenine dinucleotide phosphate reductase (NADPH) and high GST activity (Ahmed 2005). In the literature, GSH levels in *P. viridis* brachial have correlated negatively with total xenobiotic concentrations (Richardson *et al.* 2008). A negative correlation similar to that observed in *S. solida* in the study sectors (GSH *vs.* MDA concentration) has been reported by authors such as Doyotte *et al.* (1997) and Torres *et al.* (2002).

Levels of lipid peroxidation were significantly higher in tissue of the digestive glands of organisms of *S. solida* that inhabit the mouth of the Andalién River than in organisms from the mouth of the Lenga Estuary and the mouth of the Coliumo Estuary. There was a significant negative correlation between GSH concentrations *v/s* MDA concentrations in the studied sectors. Likewise, individuals that inhabit the Andalién estuary showed a lower average GSH concentration and a higher concentration of MDA. This inverse correlation between GSH and MDA concentrations has been reported by Viarengo *et al.* (1990), Doyotte *et al.* (1997) and Aloíso *et al.* (2002).

Lipid peroxidation is a complex process that occurs in plants and animals, which consists of the lipid membrane destruction and the lipid peroxides production and other sub-products such as aldehydes. Malonialdehyde (MDA) was formed from the breakdown of unsaturated fatty acids and can be used as a convenient index of lipid peroxidation (Jamil 2001).

A decline in the concentration of the non-enzymatic antioxidant GSH has been shown to harm the Deoxyribonucleic acid (DNA) of the organisms due to the oxidation of nitrogenated bases, mainly guanine by alkoxy radicals or by covalent bonds of certain damaged lipid hydroperoxide products (Akcha *et al.* 2000, Rank *et al.* 2007). Other authors have reported that xenobiotics (*e.g.*, menadione, benzo(a)pyrene, paraquat, nitrofluorantene, and hydrogen peroxide) produced by carbohydrate detoxification reactions, organochlorinated compounds, and trace metals are able to generate oxygen-reactive species and free radicals capable of inducing lipid peroxidation and oxidative stress (Doyotte *et al.* 1997, Cavaletto *et al.* 2002). Given the wide range of lipid peroxidation-inducing agents that could be present in the study area, the characteristics observed in the organisms from the Andalién estuary could be generated by complex mixtures of contaminants. Therefore, the question of this study is answered and *S. solida*, given its sensitivity, is recommended for use as a bioindicator.

Indicator species and biomarkers can be of great use in programs of environmental alertness, since they provide information on subletal effects that can be used as indicators of the need for implementation of mitigation measures.

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