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Hsp70 and p53 expression in *Gondogeneia antarctica* amphipods collected in shallow waters around the Brazilian Antarctic Station "Comandante Ferraz" (EACF), Admiralty Bay, King George Island, Antarctica

ARTHUR JOSÉ DA SILVA ROCHA, MARINA TENÓRIO BOTELHO, FABIO MATSU HASUE, MARIA JOSÉ DE A. C. R. PASSOS, CAROLINE MARGONATO CARDOSO, CAROLINE PATRÍCIO VIGNARDI, PHAN VAN NGAN & VICENTE GOMES*

Instituto Oceanográfico da Universidade de São Paulo. Praça do Oceanográfico, 191, Butantã, CEP 05508-120, São Paulo, SP, Brazil

Corresponding author: vicgomes@usp.br

Abstract. The expression of Hsp70 and p53, by applying the immunohistochemical assays, was assessed in Antarctic amphipods *Godondogeneia antarctica* from shallow waters around the Brazilian Antarctic Station "Comandante Ferraz" (EACF), in two distinct samplings. Intensity of expression was evaluated as the HSCORE of preparations from animals sampled nearby the Fuel Tanks and Sewage Treatment Outflow of the research station, in comparison to groups from Punta Plaza and Yellow Point, natural sites far from the EACF. The expression of Hsp70 was significantly higher in animals from the region of Sewage Treatment Outflow, as compared to those from the other places, for both biomonitorings. There were no significant differences between Fuel Tanks, Punta Plaza and Yellow Point. Differences in p53 expression were not significant for both samplings. This is the first attempt to study both Hsp70 and p53 proteins in Antarctic amphipod *G. antarctica* by employing immunohistochemistry technique in animals directly sampled from the environment. Results are discussed in terms of the marine pollution on the physiology of *G. antarctica*, as well as a suitable tool for physiological studies and the biomonitoring of Antarctic marine coastal habitats.

***Keywords*:** p53 expression, Hsp70 expression, Antarctic amphipods, biomonitoring

Resumo. Expressão da Hsp70 e da p53 em anfipodes Gondogeneia antarctica coletados em águas costeiras rasas, nas imediações da Estação Antártica Brasileira. A expressão das proteínas Hsp70 e p53 foi avaliada, em duas amostragens consecutivas, por meio de preparações imunohistoquímicas em anfípodes antárticos *Gondogeneia antarctica* que ocorrem em águas rasas nas cercanias da Estação Brasileira Antártica "Comandante Ferraz" (EACF). A intensidade da expressão foi avaliada pelo cálculo do HSCORE de preparações de animais coletados em frente aos tanques de armazenamento de combustível e nas proximidades do despejo de esgoto, em comparação com os coletados longe de fontes de contaminação, ou seja, Punta Plaza e Yellow Point. A expressão de Hsp70 foi significativamente mais elevada nos animais coletados em frente à saída do esgoto, quando comparada às de outros locais de coleta, em ambas as amostragens realizadas. Não houve diferenças significativas entre as preparações de animais dos outros locais. As variações da expressão da p53 em anfípodes dos diferentes locais de coleta não foram significativas. Esta é a primeira vez que essas proteínas são estudadas em *G. antarctica*. Os resultados são discutidos em relação aos efeitos da contaminação ambiental sobre a fisiologia desses animais, bem como sobre sua aplicabilidade para estudos ecológicos e de biomonitoramento.

Palavras chave: expressão da p53, expressão da Hsp70, anfípodes antárticos, biomonitoramento

Introduction

The Antarctic region is one of the most preserved environments in the world, located far from the other continents and relatively free from the massive human presence and its consequent environmental impacts. However, environmental impacts resulting from human activities in Antarctica such as fishing, tourism and research are almost inevitable. Antarctic coastal waters have been contaminated by fossil fuels and sewage outflows, resulting from the operation of ships (touristic and research vessels) as well as from around 79 near shore research stations. Hydrocarbons (Cripps & Priddle 1991), persistent organic contaminants (Weber & Goerke 2003) and others from the sewage effluents (Hughes 2004, Hughes & Thompson 2004, Montone *et al.* 2010) have been found in shallow waters and sediments of the benthic habitats nearby the areas occupied by research stations. The Brazilian Antarctic Station "Comandante Ferraz" (EACF) is a permanent establishment that houses from an average of 20 people in winter, to around 60 to 80 during the summer season (Bícego *et al.* 2009). Located at the Keller Peninsula on Admiralty Bay, King George Island, South Shetland, whose adjacent marine environment is inhabited by different organisms (Nonato *et al.* 2000, Freire *et al.* 2006), operation of the EACF is a concern regarding the environmental impacts, especially in shallow water.

Several studies have demonstrated that the marine environment around the EACF station is contaminated by a wide range of pollutants, such as hydrocarbons (Martins *et al.* 2004), polychlorinated biphenyls (Montone *et al.* 2001), as well as faecal sterols and linear alkylbenzenes (Martins *et al.* 2002, 2012). Some contaminants trigger cellular responses characterized by the activation of proteins involved with the cell stability, which may be suitable biomarkers of the environmental presence of these compounds. Chaperone Heat Shock Protein 70 (Hsp70) is known due to its ability to help other proteins to attain their functional states in cells under normal conditions. The increasing of Hsp70 expression protects the cells from hazardous consequences of stress, by assisting misfolded proteins to regain their native states and preveting the formation of proteins aggregates as well as the loss of their functions (Parsell & Lindquist 1993, Hartl 1996, Fink 1999, Ackerman *et al.* 2000, Hartl & Hayer-Hartl 2002). Several environmental stressors, such as UV radiation and different forms of pollution may trigger the synthesis of Hsp70, besides the extensive study as a protein model of thermal stress response (Kiang & Tsokos 1998). Another important component for cell stability, the tumor suppressor p53, is a protein of the cellular cycle regulation induced by a variety of stressors, such as UV radiation and pollution that cause damages to the DNA (Wahl & Carr 2001, Oren *et al.* 2002). In a genotoxicity event, p53 becomes active in the cell nucleus, where it binds to the promoter region of a target gene, leading to the cell cycle arresting or to its apoptosis (Vousden & Lane 2007).

Antarctic marine fauna of the shallow water is characterized mainly by animals with short reproductive seasons, low larval dispersal, low fecundity as well as subjected to strong and recurrent seasonal factors such as light intensity and food availability (King & Riddle 2001). The long evolutionary adaptation to a stable environment at low temperature makes these animals sensitive to perturbations on physiological factors, and also makes them suitable bioindicators for the environmental quality. *Gondogeneia antarctica*, the species selected for this study, is one of the most abundant amphipod crustacean in intertidal region of the Antarctic coastal waters. *G. antarctica* has sedentary habits, where they feed on the macroalgae and debris of the surf zone (Opalinski & Jazdzewski 1978, Jazdzewski 1993, Opalinski & Sicinski 1995). In addition, these small crustaceans are important elements of the food web, being preyed by other invertebrates (Gomes *et al.* 2009) and small fishes (Barrera-Oro & Piacentino 2007, Barrera-Oro & Winter, 2008).

The Brazilian Antarctic Program has supported studies on environmental monitoring to assess and mitigate impacts caused by the human presence to the environment and to the organisms that inhabit it (Phan *et al.* 2007, Martins *et al.* 2012). This study was aimed to the investigation of Hsp70 and p53 expression through the immunohistochemistry assays with tissues of *G. antarctica* collected in marine shallow waters at different places around the Brazilian Antarctic Research Station.

Materials and methods

Gondogeneia antarctica amphipods were captured by hand net from shallow waters of the Admiralty Bay, King George Island (62º05´S, 58º23´W), at the four different locations (Fig. 1).

Punta Plaza (PPL) and Yellow Point (YP) are located away from the EACF influence and were established as control sites to be compared with the amphipods under the possible influence of the Fuel

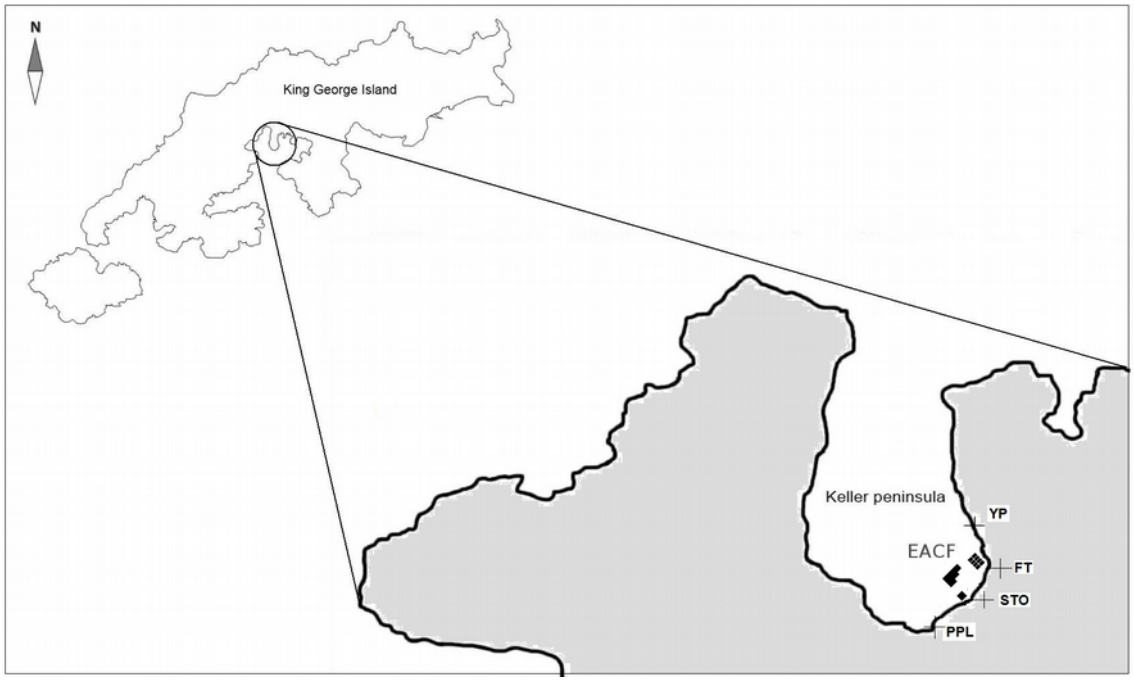

Figure 1. Map of the Keller Peninsula, King George Island, Antarctic, showing the sampling places: YP: Yellow Point; FT – Fuel Tanks; STO – Sewage Treatment Outflow; PPL – Punta Plaza.

Tanks (FT) and Sewage Treatment Outflow (STO), sites in front of the Station. Samplings were carried out twice in February 2012 and designated as biomonitoring A and B. From each site, a total of 8 individuals were sampled for the biomonitoring A and 6 individuals for the biomonitoring B. The amphipods were immediately immersed in paraformaldehyde 4% solution for 24 hours, followed by the immersion in 0.1M pH 7.4 cooled phosphate buffer solution until the processing. Samples were dehydrated in increasing solution of ethanol/water (70 to 100% ethanol), followed by 4 baths of 5 minutes in xylene and embedded in 3 X 4 cm Ever-Plast® blocs that were sliced in 5µm sections. Three sections of each organism were set in individual glass slides, previously washed in 3amino-propylethoxy-silane in order to assure a strong adherence of slices. Glass slides were dried in 60ºC from 1 to 4 hours in order to remove the excess of Ever-Plast®, rehydratedand microwaved for 20 minutes in citrate buffer (2mM citric acid and 9mM trisodium citrate, pH 6.0) for the antigen recovery.

Immunostaining was performed by employing a secondary biotinylated antibody, streptavidin-biotin-peroxidase complex solution (Dako Kit K0679), chromogen DAB (Dako K3464) and also a protein block solution DAKO X0909 (Dako North America, Inc., Carpinteria, CA, USA) for indirect immunoperoxidase method (Ramos-Vara 2005). Glass slides were also counterstained with Harry´s hematoxylin-eosin solution (HE) in order to carry a structural morphological description of *G. antarctica* tissues (Machado *et al.* 2012). Hsp70 and p53 proteins were detected in slides marked as positive to mouse monoclonal Hsp70 (BRM-22, Sigma®) and p53 (BP53-12, Sigma®) primary antibodies, at the respective 1:10000 and 1:5000 concentrations in citrate buffer, after 20 hours of cooled incubation. The immunoreaction conditions were based on immunostaining procedures previously tested at different antibodies concentrations incubated for 20 hours. Negative control slides were incubated with citrate buffer without primary antibodies. All the slides were examined and photomicrographs were taken under a light microscope. Four intensity levels of the immunological stainings were established (Fig. 2) and classified by employing the HSCORE in accordance to Flanagan *et al.* (2008), with modifications.

The HSCORE is a semiquantitative analysis related to the intensity and the percentage of stained cells and is calculated as follows: HSCORE = $\sum Pi(i)$, where the i is the intensity of staining (0 = none, 1 = weak; 2 = moderate; 3 = strong) and Pi is the percentage of stained cells in each intensity, ranging from 0 to 100%. From the values of the glass slides, a mean value was established for each individual and means of HSCOREs and standard errors (±SE) were calculated for each site and

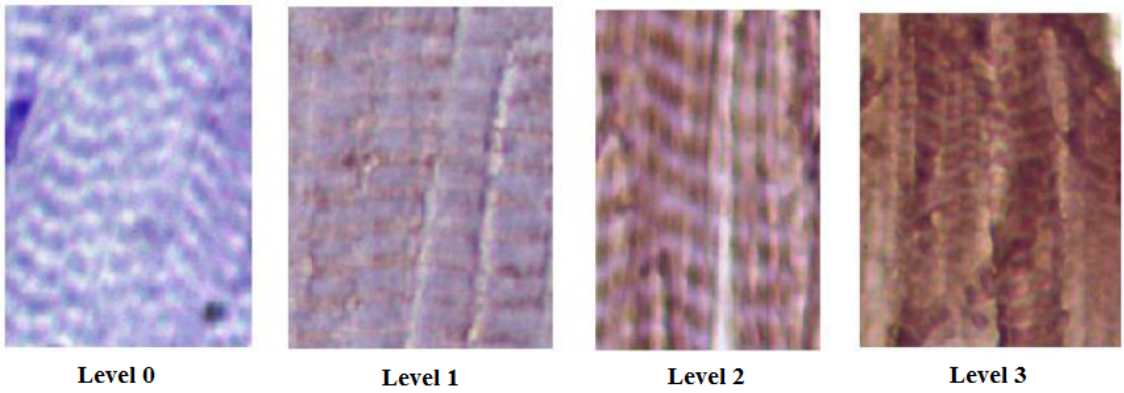

Figure 2. Levels of intensities of the immunological staining for Hsp 70 and p53 proteins in muscular tissue of *Gondogeneia antarctica* amphipods.

biomonitoring. Means (±SE) HSCOREs were tested for homogeneity of variances by the Levene´s test and differences between sites and biomonitorings determined by the analysis of Variance (ANOVA) and Tukey HSD post-hoc test ($p<0.05$).

Results and Discussion

Environmental monitoring of Antarctic regions occupied by signatory countries is a goal of the Antarctic Treaty (Santos *et al.* 2006, Phan *et al.* 2007, Gomes *et al.* 2009, 2012) and biomonitoring studies require systems that quantitatively and qualitatively describe the environment (Rocha *et al.* 2015). Organisms that are in direct contact with pollutants may be suitable bioindicators (Rajaguru *et al.* 2003).

The ability to predict the vulnerability of a species to stress is best achieved at the molecular level, as sub-lethal effects across a range of functions can be quantified (Clark & Peck 2009). Hsps, specially the 70 kDa (Hsp70), and p53 are proteins evolutionary conserved in different biological groups (Lindquist & Craig 1988, Banni *et al.* 2009). Despite that the induction of Hsp70 is generally related to external temperature rise above the organism optimum (Lindquist 1986), its expression in marine organisms can be induced by other factors such as heavy metals (Kim *et al.* 2014), ultraviolet radiation (Won *et al.* 2015), polycyclic aromatic hydrocarbons (Liu *et al.* 2015) as well as pathogens (Baruah *et al.* 2014), as usually found for the p53 family (Dahms & Lee 2010, Qian *et al.* 2014, Kim *et al.* 2015).

The immunohistochemistry methodology is a feasible technique for the study of cellular proteins in marine vertebrates and invertebrates as well (Papo *et al.* 2014, Cardoso *et al.* 2015, Xu *et al.* 2015). In this study, previously to the immunohistochemistry staining, a broad assessment of the inner morphology of *G. antarctica* was performed in order to focus the study in structures well characterized (Fig. 3).

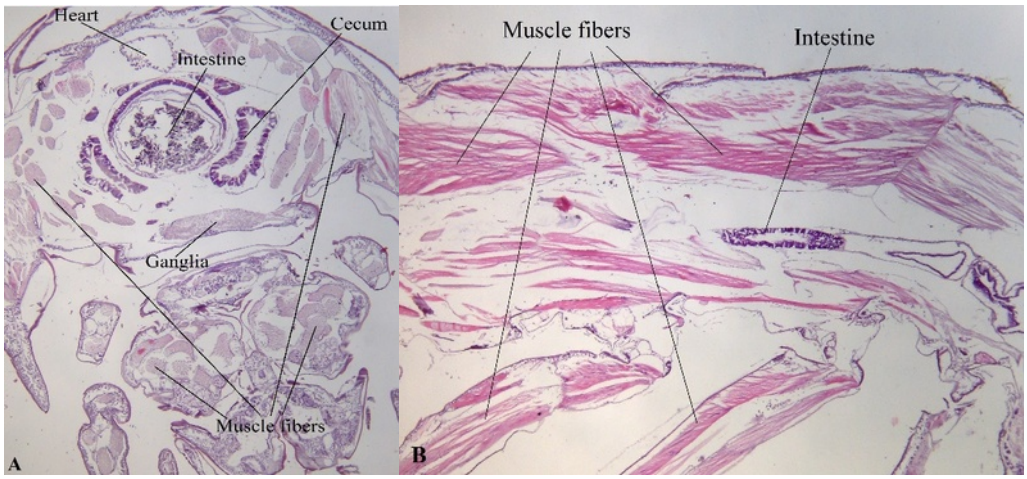

Figure 3. Cross section (A) and longitudinal section (B) of *Gondogeneia antarctica*, showing different HE stained tissues.

Different organs such as cecum, heart, ganglia and intestines are HE stained, as well as the fibers of different muscular groups. Since muscular tissue covers a wide area in the slides, it was chosen for the analysis of both Hsp70 and p53 expression in *G. antarctica* captured from different places around the EACF research station

Negative controls (slides without monoclonal antibody) did not exhibit immunohistochemical staining, indicating that the methodology was well fitted to the *G. antarctica* tissue, since no staining means no protein expression, as compared to those slides where both Hsp70 and p53 were expressed. They were very similar to those found in a previous study with the teleost fish Florida Pompano *Trachinotus carolinus*, whose patterns of immunoexpressions were tested against those in the heart tissue of *Ratus norvegicus* as experimental quality control (Cardoso *et al.* 2015). These findings confirm the efficiency and specificity of the antibodies for both, Hsp70 and p53 proteins and support the theory of phylogenetically conservative traits in *G. antarctica*. The analyses of both proteins immunoreactions were efficiently performed by the HSCORE (Flanagan *et al.* 2008), a methodology widely employed in cell research, which relative simplicity does not demand sophisticated software for its procedure.

Results of the Hsp70 immunoreactions from the biomonitorings are shown in the Figure 3. The mean HSCORE was significantly higher at the STO site, as compared to the others locations, for both biomonitorings. There were no significant differences in the Hsp70 expression between PPL, YP and FT sites. Shallow waters around the EACF are usually contaminated by high levels of petroleum hydrocarbons and other organic compounds (Bícego *et al.* 1996, 1998, Martins *et al.* 2004), as well as by inorganic compounds, such as heavy metals and metalloids (Santos *et al.* 2005, Ribeiro *et al.* 2011), released during the EACF operations. Different hydrocarbon compounds have been found at the sea bottom as well as in the water samples nearby the fuel tanks, due to direct input by fuel leakage or from the combustion of fossil fuels (Bícego *et al.* 2009). Table I presents levels of different organic compounds in sediments of the shallow waters around the Brazilian Antarctic Research Station obtained in some studies along the 12 years of observation.

Elevated levels of fecal sterols were found nearby the EACF sewage effluent outflow, although lower than those measured before the 2005-2006, when the sewage treatment system started to operate after improvements (Martins *et al.* 2012). However, even after the improvements on the sewage treatment system of the station, alkylbenzesulphonates are still high relative to the more pristine areas, what may help to explain the significant Hsp70 expression in *G. antarctica* of the STO as compared to the other places (Fig. 4).

The lowest values of contaminants (Table I) correspond to those found in areas far from the influence of the EACF, such as Punta Plaza and Yellow Point, both places where the Hsp 70 of *G. antarctica* presented the lowest expression as well. This result is similar to those found for the genotoxicity of *G. antarctica*, as the lowest DNA damages were found in hemocytes of animals from Punta Plaza and Yellow Point (Rocha *et al.* 2015), both areas away from the EACF influence. PAHs are known to induce oxidative stress (Liska 1998) and the consequent activation of chaperone proteins may counteract those effects by stabilizing other proteins involved with detoxification processes (Gupta *et al.* 2010).

Table I. Range of sewage organic compounds and hydrocarbons in sediments of the shallow waters (0-10 m depth) in front of the Brazilian Antarctic Research Station EACF and neighbor areas.

Reference	Faecal sterols $\mu g\text{-}g^{-1}$	LABs $ng\text{-}g^{-1}$	Σ-AHs $\mu g\text{-}g^{-1}$	Σ-PAHs $ng\text{-}g^{-1}$
Bícego *et al*., 1998	n.a	n.a.	0.14 - 0.58	1.00 - 32.0
Martins *et al*., 2002	0.21 - 10.4	<0.60 - 11.8	n.a	n.a.
Martins *et al*., 2004	n.a	n.a.	0.15 - 13.28	9.45 - 270.5
Montone *et al.*, 2010	0.01 - 0.95	1.00 - 23.0	n.a	n.a.
Martins *et al*., 2012	0.01 - 0.17	1.00 - 46.5	n.a	n.a.

LABs: linear alkylbenzenes; Σ-AHs: total aliphatic hydrocarbons; Σ-PAHs: total polycyclic aromatic hydrocarbons; n.a.: not available

Surprisingly, the expression of Hsp 70 in *G. antarctica* collected from shallow waters near the fuel tanks were similar to the mean values of those animals from Punta Plaza and Yellow Point, for both biomonitorings (Fig. 4). Perhaps, the effects of hydrocarbons at the present levels of contamination in this area are not enough to cause the expression of chaperone proteins, in opposition to the results found by our studies on genotoxicity (Rocha *et al.* 2015). Different regulatory proteins could be better biomarkers of hydrocarbons than Hsp70.

Over expression of Hsp70 proteins have also been described in aquatic organisms exposed to xenobiotics or from areas impacted by industrial and urban pollution (Hamer *et al.* 2004, Rhee *et al.* 2009), including heavy metals (Kim *et al.* 2014, Rajeshkumar *et al.* 2011), chlorinated compounds (Lawrance *et al.* 1998, Morales *et al.* 2014) and endocrine disruptive chemicals (Snyder et *al.* 2001, Planelló *et al.* 2008, 2011). Although there were no consistent data regarding the aforementioned xenobiotics contamination of the shallow waters from the sewage outflow of the EACF, each one of them may be related to the significant Hsp70 expression in *G. antarctica* collected in this place (Fig. 4).

The Hsp70 expression in Antarctic marine organisms seems not to follow a simple rule; although lots of taxa have the gene, some species can just express the protein but with no regulation, others have lost the gene and others express the protein and also have a regulatory mechanism (Clark & Peck 2009). An Antartic gammarid (*Paraceradocus gibber*), for example, have inducible Hsp70 gene, but it has no significant up regulation with temperature variation (Clark *et al.* 2008). Even in the sub-Antarctic region, a simple rule for Hsp70 expression is not applied. Hsp70 was not detectable in amphipod *Hyale hirtipalma* and showed up regulation in the isopod *Exosphaeroma gigas* with temperature variation (Clusella-Trullas *et al.* 2014). *G. antarctica* expressed this protein and apparently regulated the expression, showing a higher staining in STO.

The expression of p53 proteins, usually associated to genome stability or cell cycle arrest, has been described in a variety of marine invertebrate, such as *Mytilus* sp. exposed to PAHs (Banni *et al.* 2009), Pacific white shrimp *Litopenaeus vannamei* exposed to heavy metals (Qian *et al.* 2014) as well as in benthic copepod *Trigiopus japonicus* (Kim *et al.* 2015), sea urchin embryos and cod larvae (Dahms & Lee 2010) under the UV radiation. In spite of the positive p53 immunoreactions of *G. antarctica* in animals sampled from the four shallow water places around the EACF, there were no significant differences in the mean HSCORE, for both biomonitorings (Fig. 5).

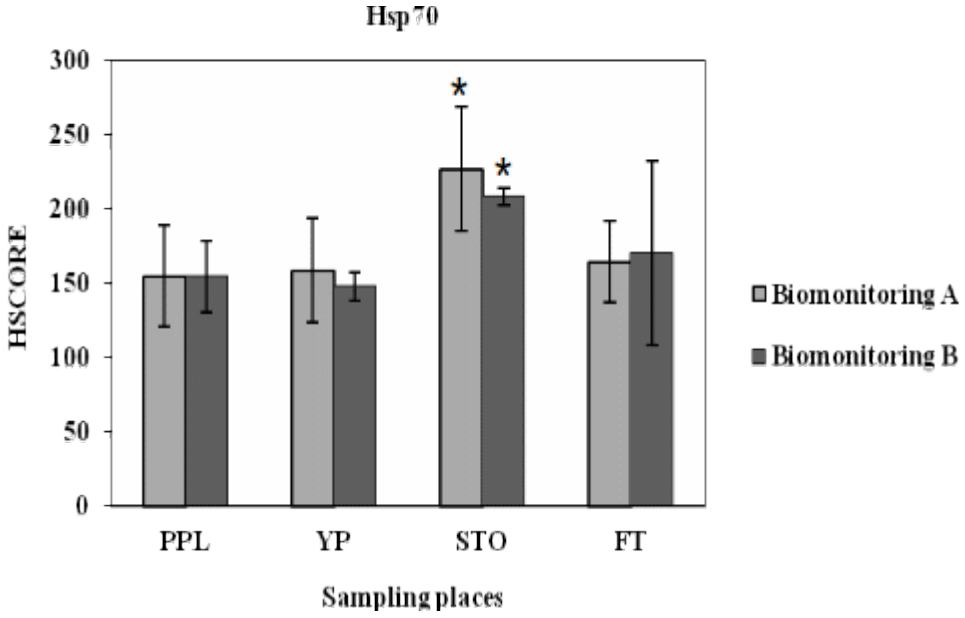

Figure 4. Mean (±SD) HSCORE of the Hsp70 protein immunoreaction in muscular tissue of *Gondogenia antarctica* amphipods, collected from different sampling places around the EACF research station. * denotes significant differences in the STO as compared to the others sampling places, in each biomonitoring ($p<0.05$).

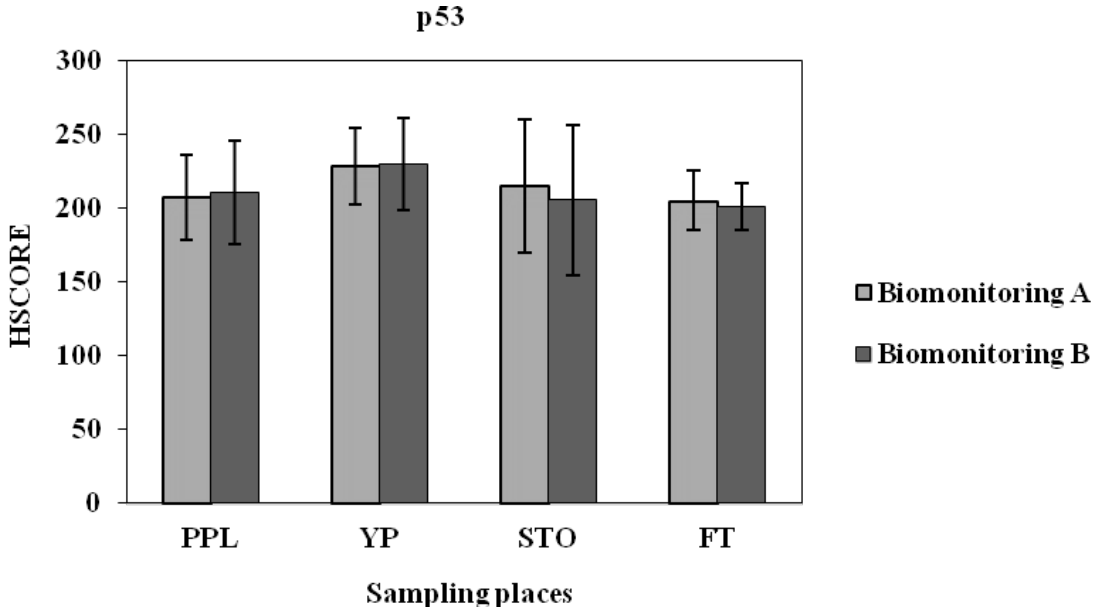

Figure 5. Mean (±SD) HSCORE of the p53 protein immunoreaction in *Gondogenia antarctica* amphipods, collected from different sampling places around the EACF research station.

Expression of p53 in muscle tissue of *G. antarctica* was conspicuous and promptly identifiable. Nevertheless, we expected some modulation of the p53 expression, that would mirror the significant results obtained in our previous work on DNA damage of *G. antarctica* sampled at shallow water areas in front of the fuel tanks and sewage treatment outflow places, detected by the comet assay (Rocha *et al.* 2015). Despite the aforementioned significant DNA damage, perhaps an acute contamination high enough to trigger an enhancement of the normal expression of p53 in *G. antarctica* was not occurring.

As a first attempt, we satisfactorily achieve the basic principles to study both Hsp70 and p53 proteins in Antarctic amphipod *G. antarctica* by employing immunohistochemistry technique in animals directly sampled from the environment. Nevertheless, this subject deserves more attention by means of specific experimentation, such as those under the controlled conditions of laboratory in order study the cause-effect on these proteins as biomarkers of different environmental factors that impact the marine fauna of the Antarctica coastal waters.

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