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Published online: 25 Mar 2010.

To cite this article: Joanna Kolasinski, Dorothée Taddei, Pascale Cuet & Patrick Frouin (2010): AChE and EROD activities in two echinoderms, Holothuria leucospilota and Holoturia atra (Holothuroidea), in a coral reef (Reunion Island, South-western Indian Ocean), Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 45:6, 699-708

To link to this article: http://dx.doi.org/10.1080/10934521003648917

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AChE and EROD activities in two echinoderms, *Holothuria leucospilota* and *Holoturia atra* (Holothuroidea), in a coral reef (Reunion Island, South-western Indian Ocean)

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Laboratoire d'Ecologie Marine, Université de La Réunion, La Réunion, France

AChE and EROD activities were investigated in two holothurian species, *Holothuria leucospilota* and *Holoturia atra*, from a tropical coral reef. These organisms were collected from 3 back-reef stations, where temperature and salinity were homogeneous. The activity levels of both AChE and EROD varied significantly between the two species, but were in the range of values determined in other echinoderm species. AChE activity levels were higher in the longitudinal muscle than in the tentacle tegument. Among the several tissues tested, the digestive tract wall exhibited higher EROD activity levels. Sex did not influence AChE and EROD activity levels in both species. Animal biomass and EROD activity levels were only correlated in the tegument tissue of *H. atra*, and we hypothesize a possible influence of age. EROD activity did not show intraspecific variability. A significant relationship was found between AChE activity and Cuvierian tubules time of expulsion in *Holothuria leucospilota*. Individuals collected at the southern site presented both lower AChE activity levels and Cuvierian tubules time of expulsion, indicating possible neural disturbance. More information on holothurians biology and physiology is needed to further assess biomarkers in these key species. This study is the first of its kind performed in the coastal waters of Reunion Island and data obtained represent reference values.

**Keywords:** AChE, EROD, Holothuria, coral reef, Reunion Island, South-western Indian Ocean.

**Introduction**

Increasing human impacts on coastal zones, such as degradation of marine habitats, raise environmental concerns and growing attention.[1] Tropical marine environments are unfortunately less monitored than temperate ones regarding pollution impacts. Atmospheric concentrations of persistent organic pollutants (POPs) over the open Indian Ocean are in a similar range to those reported for remote land-based locations in the more industrialized northern hemisphere.[2] Various organic pollutants were detected in almost all tuna specimens from southern tropical areas in a recent global monitoring,[3] indicating widespread contamination by these compounds in the marine environment. The tropical zone of the Indian Ocean has, up to now, received little attention from researchers with reference to levels of pollutants or toxicity biomarker activity in marine organisms.

Over the last few years, the wide range of molecules released into the marine environment has led to the development of analyses based on biomarkers.[4] The use of a biological response to toxic stress may provide rapid and sensitive means for evaluating exposure to environmental contaminants in ecosystems where the effects of stressors are unknown.[5] Investigations of acetylcholinesterase (AChE) and ethoxyresorufine-\(O\)-deethylase (EROD) activities are widely used with respect to specific exposure biomarkers of pesticides and organic contaminants by international organizations and environmental agencies. AChE is responsible for the degradation of the neurotransmitter acetylcholine, regulating its proper levels in the central and parasympathetic nervous systems, neuromuscular junctions, and sympathetic synapses. Estimation of AChE activity inhibition is used as an indication of neurotoxic effects and has been proposed as evidence of organophosphate and carbamate poisoning.[6]

The cytochrome P450 (CYP450)-dependent monoxygenase system, also called mixed function oxygenases (MFO), is responsible for the biotransformation of endogenous and exogenous lipophilic compounds.[7] The induction of CYP450, measured catalytically as EROD activity, is now widely used to assess environmental pollution as specific marker of exposure to organic contaminants such as
polycyclic aromatic hydrocarbons (PAHs), planar polychlorinated biphenyls (PCBs) or dioxins in monitoring programs on mammals and fish. Most studies describing the use of AChE levels in marine organisms as a contamination biomarker have mainly dealt with fish[8] and mollusks.[9] Little is known for the phylum Echinodermata[10−14] and among them holothuroids, which have not been tested yet. CYP450 enzymes belong to a widely distributed protein super-family with several families and subfamilies and their presence has been confirmed in a number of marine invertebrate species.[15,16] In Echinoderms, sequencing of the sea urchin Stronglylocentrotus purpuratus genome has revealed 120 different P450 genes,[17] but catalytic functions, as yet, have not been linked to specific P450s. Several studies have shown the presence of P450 catalyzed enzymatic reactions in tissues of at least three echinoderm classes: asteroids (Asterias rubens and Marthasterias glacialis), echnoids (Stronglylocentrotus sp.) and holoturians (Holothuria forskali), see review by den Besten.[18]

In the present study, we determined AChE and EROD activities in several tissues of two holothurian species Holothuria leucospilota and Holothuria atra (Echinodermata) from a coral reef in Reunion Island (Southwestern Indian Ocean), and estimated variability in the populations. In this reef, the two species are the dominant sediment deposit-feeders by biomass.[19] Holothurians are interesting test organisms because of their worldwide distribution, from tropics to polar regions, and their important contribution to the sediment organic matter recycling. As far as we know, this is the first study that investigates biomarker activities in benthic macroinvertebrates from coral reef environments. The holothurians analyzed have been sampled few weeks before the Chikungunya epidemic broke out at Reunion Island in April 2005. Organophosphates and pyrethroid insecticides were used then as vector controls[20] and may have impacted non-target species. Therefore, the present data are reference values that may be useful to assess impact of control treatment on communities.

**Materials and methods**

**Study site**

Reunion Island is situated in the South-western Indian Ocean (21°07’S, 55°32’E), 800 km east of Madagascar (Fig. 1), and is characterized by a tropical oceanic climate. The sampling regime was undertaken in March 2005 during the summer season which extends from January to April, with sea surface temperature ranging from 27.4°C to 28.0°C.[21] The study was carried out at the La Saline fringing reef (4 km long, 500 m maximum width), located on the west coast of the island (Fig. 1). Holothurian specimens were collected at three back-reef sites (50m from the line coast), from south to north: Trou d’Eau (TE), Planch’Alizés (PA) and Club Med (CM). The depth varied from 1.0 to 1.5 m, according to tidal level. Soft substrata are composed of coarse sand scattered with coral fragments. The La Saline reef is surrounded by mouths of the “Hermitage” (north) and “Trois-Bassins” (south) streams. The surrounding land (30 km) is used primarily for agriculture, including sugarcane and market gardening productions.

**Sampling strategy**

Seven specimens of Holothuria leucospilota and Holothuria atra were hand-captured at each site (except in TE where only 5 H. atra individuals were collected due to low densities). Only individuals with good health signs[22] were collected. Holothuria leucospilota possess Cuvierian tubes which are conspicuous intracoelomic caeca found in some species of aspidochirote holothurians (not present in H. atra). This defensive organ is discharged through the anus when the animal is stressed. Cuvierian tubes time of expulsion after capture was recorded for each H. leucospilota individual in order to assess general stress status.

Longitudinal body muscle containing large neurons and tegument of tentacles from buccal area were selected for AChE analyses. Dorsal body tegument, digestive tract wall and respiratory tree tissues were sampled for EROD
analyses. Tissues were sampled in triplicate for each individual. Animals were sacrificed in the field, tissues were dissected and intestine content was removed. Tissues were rinsed with deionized water and immediately placed into liquid nitrogen (−196°C). Samples were then stored at −80°C in the laboratory until analyses. Gonadal maturity stage was determined by direct microscopic observation of formalin-preserved gonads. Size (total length, TL) was recorded for each individual. Obtaining accurate morphological and mass measurements of holothurians is difficult due to their irregular form, body contractibility and high water content in tissue. Expressing biomass in ash-free dry weight (AFDW) is more reliable for macro-invertebrates due to their irregular form, body contractibility and high water content in tissue. Expressing biomass in ash-free dry weight (AFDW) is more reliable for macro-invertebrates since tissue water content and non-organic components such as spicules are taken into account. Dry weight (DW) was obtained by placing animals in an oven at 60°C until constant weight and ash content was determined by burning dried animals in a muffle furnace at 550°C for 3h. In situ temperature and salinity were recorded at the sediment-water interface using an YSI 6920 multiparameter probe.

**AChE assay**

Around 1g of tissue maintained in an ice bath was ground with a Potter-Elvenyhem homogenizer in a 0.02 M pH7 phosphate buffer + 0.1% Triton X100 (1/4 v/w). The homogenates were then centrifuged at 10 000 × g for 20 min at 4°C. The supernatant, containing the cytosol, endoplasmic reticulum, Golgi apparatus and cytosolic proteins, was removed and used to determine the AChE activity. AChE activity was determined according to the method of Ellman et al., with acetylthiocholine iodide (AcSCh) as a substrate and 5,5'-dithio-bis-2-nitrobenzoate (DTNB) as a reagent. AcSCh is the most useful substrate to assess AChE in sea urchins (Echinodermata) Paracentrotus lividus. The released thiocholine reacts with DTNB to produce 5-thio-2-nitrobenzoate (TNB), which absorbs at 412 nm. For this purpose, 40 µl of supernatant were used in the reaction together with 1360 µL phosphate buffer pH 7 + 0.1% Triton X100, 80 µL 0.01 M DTNB and 40 µL 0.1 M AcSCh. Enzymatic activity was measured for 5 min in a UV spectrophotometer and read continuously for 3 min. For each homogenate sample, three subsamples were assayed. Bradford’s method was used for quantitative analysis of supernatant proteins, with bovine serum albumine (BSA) as the standard. Specific activity is expressed in nanomol of AcSCh, hydrolyzed per minute per milligram of protein. All assays were performed in triplicate.

**EROD assay**

The principle of the assay is based on the hydrolyzation of the substrate ethoxyresorufin, according to Burke and Mayer. Tissues (~1 g) were homogenized in a 100 mM phosphate buffer with pH 7.4, 1:4 (v/w) Na2HPO4, and KH2PO4 containing a 0.2 mM PMSF protease inhibitor cocktail. The homogenate obtained from the reaction was centrifuged at 10 000 × g for 20 min at 4°C and run through a second centrifuge at 10 000 × g for 1h with 1 mL of 100 mM phosphate buffer, containing 20% glycerol. Resuspended microsomes were stored at −80°C until subsequent analysis. The isolated microsomal fraction was then used to determine EROD activity using spectrofluorimetric methods. 75 µl of the microsomal fraction were added to 1350 µL of 100 mM phosphate buffer (pH 7.4), 75 µl of 2 µM 7-ethoxyresorufin in DMSO, and 225 µL 1.9 mM NADPH. The reaction was initiated by the addition of NADPH and the fluorescence was monitored for 6 min by a spectrofluorometer (Turner 700) using 520 nm (excitation) and 590 nm (emission) filters. A known amount of pure reference of resorufin was added as an internal standard to the reaction mixture. EROD activity was measured as picomol per minute per milligram of protein. Total protein concentration was measured by the Bradford method using a BSA standard. All assays were performed in triplicate.

**Statistical analyses**

Statistical analyses were performed using Statistica 7.0 software (StatSoft, USA). Data were expressed as a mean ± standard deviation. Differences in species biometric parameters and spatial variability in water temperature and salinity were examined using ANOVAs. Non-parametric tests were used when data did not fulfil homoscedasticity requirements. Tissue AChE and EROD activity levels were compared using Student t-tests. Influence of species biomass on AChE and EROD activity levels were tested using Pearson’s correlation and ANOVAs. Data were statistically contrasted in spatial analyses to consider the potential effect of holothurians biomass: differences among sampling sites were examined using ANCOVAs with AFDW as covariate. Multivariate analysis was performed on both AChE and EROD responses for every tissue. A similarity matrix based on Bray-Curtis coefficient was classified by hierarchical agglomerative clustering using Unweighted Pair Group Mean Arithmetic (UPGMA) and multi-dimensional scaling (MDS) linking methods. Calculations were performed with PRIMER 6.0. The level of significance was established at P < 0.05 for statistical tests.

**Results and discussion**

Despite biomarkers having been used for over 20 years in aquatic organisms, data on biomarker activity levels in echinoderms are still limited and such assessment is particularly true for holothurians. This study reports preliminary data on AChE and EROD activity levels in tissues of two native holothurian species from a tropical coral reef.
**AChE activity levels**

Average AChE activity levels measured in muscle and tentacle tegument tissues of *Holothuria leucospilota* and *Holothuria atra* are reported in Figure 2a and Figure 2b, respectively. There were significant differences in AChE activity levels regarding tissue type in both species (Table 1). Tentacle tegument tissue exhibited lower AChE activity levels (mean value of 2.6 ± 2.4 nmol min⁻¹ mg protein⁻¹ in *H. leucospilota* and 0.7 ± 0.8 in *H. atra*) compared to muscle tissue (48.3 ± 15.7 in *H. leucospilota* and 11.0 ± 2.0 in *H. atra*). There were significant interspecific differences in AChE activity levels in muscle and tentacle tegument tissues (Table 2). AChE activity levels were 4 times higher in *Holothuria leucospilota* than in *Holothuria atra*. Even though there are no available data on holothurian AChE activity to compare with our data, levels were in the range of values reported for other echinoderm species.

Activity levels measured in the pyloric caeca of the starfish *Asterias rubens* ranged from 2.9 ± 0.5 to 31.7 ± 8.2 nmol min⁻¹ mg protein⁻¹,[11] and from 42.7 to 65.0 in the sea urchin *Paracentrotus lividus* specialized tissues such as muscles of Aristotle’s Lantern, ambulacra podia and intestine.[14] In aquatic organisms, there is considerable diversity in the biochemical properties and distribution of cholinesterases, as well as in their sensitivity to anticholinesterase agents that can influence activity values between taxa.[27,28] These interspecific variations were proposed to be ascribed to phylogeny in marine fish,[29,30] and data obtained in this study add knowledge for further similar comparisons in marine invertebrates.

Echinoderms are among the most important groups of marine metazoans from the point of view of evolution and ecology. Nevertheless, their nervous system has been little studied. Basic information about the anatomical location of neuron and fibre expressive neurochemicals and about the neurochemical nature of neuronal elements are still needed.[31] However, the widespread sensitivity to acetylcholine, adrenaline and related substances suggests that chemical systems of junctional transmission occur in this phylum. Recently sea urchin coelomocytes have been successfully used to monitor the effects of heavy metals on the expression of stress markers[32] and the effects of cold temperature stress on AChE activity.[12] In this study, AChE activity levels were higher in longitudinal muscle than in the tegument of buccal tentacle. Even tentacle tegument tissue contain nerve plexi involved in the control of tensile changes[33] and chemoreception.[34] AChE levels measured were too low to detect the intraspecific variability observed in muscle. Immunocytochemical and pharmacological studies have provided evidence for remarkable excitatory cholinergic component to the motoneurons of the longitudinal muscle of holothurians.[35] From an evolutionary point of view, γ-aminobutyric acid (GABA) modulation of acetylcholine responses in the holothurian longitudinal muscle is similar to the dual GABAergic–cholinergic innervation of the vertebrate nerve plexus–ileum muscle.[36] Hence, we recommend the use of muscle tissue for further AChE experiments in these species.

**EROD activity levels**

Average EROD activity levels are presented in Figure 3a for *H. leucospilota* and Figure 3b for *H. atra*. Activity levels measured in the digestive tract wall tissue were 3 times

**Table 1. Student t-test comparisons of AChE and EROD activity levels in different tissues of *Holothuria leucospilota* and *Holothuria atra.*

<table>
<thead>
<tr>
<th>Tissue comparison</th>
<th><em>H. leucospilota</em></th>
<th><em>H. atra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle/tentacle tegument</td>
<td>20.96 (&lt;0.001)</td>
<td>13.17 (&lt;0.001)</td>
</tr>
<tr>
<td>Digestive tract wall/tegument</td>
<td>3.33 (0.002)</td>
<td>4.54 (&lt;0.001)</td>
</tr>
<tr>
<td>Digestive tract wall/respiratory tree</td>
<td>3.95 (&lt;0.001)</td>
<td>0.72 (0.079)</td>
</tr>
<tr>
<td>Tegument/respiratory tree</td>
<td>3.78 (&lt;0.001)</td>
<td>2.82 (0.006)</td>
</tr>
<tr>
<td>EROD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue comparison</td>
<td><em>H. leucospilota</em></td>
<td><em>H. atra</em></td>
</tr>
<tr>
<td>Muscle/tentacle tegument</td>
<td>20.96 (&lt;0.001)</td>
<td>13.17 (&lt;0.001)</td>
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</tr>
<tr>
<td>Tegument/respiratory tree</td>
<td>3.78 (&lt;0.001)</td>
<td>2.82 (0.006)</td>
</tr>
</tbody>
</table>

*Student t-values, with *P* values in parenthesis. Values in bold indicate significant differences.

**Table 2. Interspecific (ANOVARs) and site (ANCOVARs, AFDW as covariate) differences in AChE and EROD activity levels measured in *Holothuria leucospilota* and *Holothuria atra.*

<table>
<thead>
<tr>
<th>Species</th>
<th>AChE</th>
<th>EROD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle</td>
<td>Tentacle tegument</td>
</tr>
<tr>
<td><em>H. leucospilota</em></td>
<td>29.19 (&lt;0.001)</td>
<td>9.79 (0.002)</td>
</tr>
<tr>
<td><em>H. atra</em></td>
<td>15.62 (&lt;0.001)</td>
<td>1.24 (0.340)</td>
</tr>
</tbody>
</table>

*H and *P*-value in parenthesis. Bold *P*-value indicate significant effect.
higher in *H. leucospilota* than in *H. atra*, but no significant interspecific differences were observed in tegument and respiratory tree tissues (Table 2). The three tissues presented distinct activity levels in *H. leucospilota* (*P* < 0.01 in each case, Table 1): digestive tract wall exhibited the highest activity levels (mean of 44.4 ± 40.2 pmol min⁻¹ mg protein⁻¹), followed respectively by respiratory tree (6.6 ± 8.0) and tegument (1.6 ± 1.5). EROD activity measured in *H. atra* digestive tract wall (13.8 ± 17.1 pmol min⁻¹ mg protein⁻¹) and respiratory tree (10.5 ± 11.2) presented similar levels (*P* > 0.05, Table 1). Levels in the two latter tissues differed from those measured in tegument (0.9 ± 0.8, *P* < 0.01, Table 1).

CYP occurs as a super-family of hundreds of genes and isoenzymes involved in protection from and repair of damage that have been largely studied in recent years with respect to molecular characteristics and phylogenetic relationships.[17,37,38] The cytochrome P450 and b5, and the MFO system-associated NADH-ferricyanide reductase, NADH-cytochrome-c reductase, NADPH-cytochrome c reductase and benzo[a]pyrene hydroxylase (BPH) activities were present in microsomal fractions of the haemal plexus of *Holothuria forskali* and in pyloric caeca of *Asterias rubens* and *Marthasterias glacialis*, indicating that these echinoderm species have the same major MFO system components as found in other invertebrates and vertebrates.[18] Activity levels measured in *H. leucospilota* and *H. atra* were slightly lower than values found for other species of the phylum. Mean values of 40 and 76 pmol min⁻¹ mg protein⁻¹ were measured in *H. forskali* and *M. glacialis* respectively, and from 25 to 128 pmol min⁻¹ mg protein⁻¹ in *A. rubens*.[18] Differences observed between the two species were only detected in the digestive tract wall tissue, which exhibited the highest activity, what was consistent with the tissue specific localization of CYP-MFO system in invertebrate phyla. The MFO system has a wide tissue distribution, but highest levels towards xenobiotics are generally found in tissues concerned with the processing of food, such as hepatopancreas of crustaceans, intestine of polychaetes, and digestive gland of molluscs or pyloric caeca of asteroids.[39]
Holothurians digestive tract wall tissue is constituted by a mucosal epithelium capable of secreting enzymes, associated with the haemal system.\textsuperscript{[40]} Thus, it plays a major role in holothurian metabolism, responsible of nutrient and sugar transport, and potentially acts as a protection barrier against ingested sediment containing aggressive chemicals (microbial products, phytotoxins and other biogenically derived PAHs). MFO system induction towards planar PAHs and PCBs was observed in the asteroid \textit{A. rubens} during in vivo experiments.\textsuperscript{[41,42]} However, the known aryl hydrocarbon receptor (AHR) homologs, which are involved in the regulation of some CYP genes, do not bind typical ligands of the vertebrate AHR,\textsuperscript{[43]} suggesting an important distinction in AHR functions that is still not well understood. Characterization of holothurians P450 genes function in future research will increase our understanding of basic physiology and biochemistry of these invertebrates and should provide indications for discerning interference between endogenous processes and environmental contamination effect.

\textit{Influence of biometry and spatial variability}  

Variations of biotic parameters such as size, age, sex and gonadal maturity are known to influence biological markers.\textsuperscript{[44,45]} The use of biomarkers in field studies requires knowledge on their natural variability related to biometric and physiological parameters.\textsuperscript{[28]} Compared to other marine invertebrates, little is known about these parameters for holothurians in their natural environments, mainly due the difficulty of manipulation and long term tagging of these animals.\textsuperscript{[46,47]} Producing experimental conditions for field populations is also problematic since nutrition sources are still poorly identified and environmental conditions are known to influence their reproduction mode (sexual/asexual).\textsuperscript{[48]} Such factors could induce strong bias when monitoring physiological and biochemical processes and should be further characterized for holothurians. Here we investigated AChE and EROD activity levels in \textit{Holothuria leucospilota} and \textit{Holothuria atra} individuals from the back-reef compartment of a small coral reef which represents a natural mesocosm.

Biometric variables are presented in Table 3. No significant differences in both TL (ANOVA, $F = 0.18$, $p = 0.835$) and AFDW ($F = 1.26$, $p = 0.302$) were observed in \textit{H. leucospilota} individuals from TE, PA and CM sites. In contrast, significant differences in both parameters were found for \textit{H. atra} (Kruskal–Wallis: TL, $H = 15.67$, $P < 0.001$; AFDW, $H = 16.57$, $P < 0.001$). Newman-Keuls post-tests revealed differences between \textit{H. atra} individuals from TE site (means of 29.8 ± 7.3 cm TL and 9.3 ± 3.5 g AFDW) and those collected at PA (14.1 ± 2.8 cm and 1.9 ± 1.1 g) and CM (17.2 ± 4.7 cm and 2.4 ± 0.8 g). The two species differed both in TL (Kruskal–Wallis, $H = 9.82$, $P = 0.002$) and AFDW (H = 9.80, $P = 0.002$). There was no difference in biometric parameters (Kruskal–Wallis: TL, $H = 0.24$, $P = 0.623$; AFDW, $H = 0.01$, $P = 0.935$) between the two species in TE site (Table 2). Since TL and AFDW are significantly correlated in both species ($P < 0.05$, Fig. 4), AFDW is used to test further the influence of animal biometry.

All individuals present the same stage III mature gonads, and no significant effect of sex on activity levels was found ($P < 0.05$ in each case). There were no relationships

\textbf{Table 3.} Biometric variables and Cuvierian tubules time of expulsion of \textit{Holothuria leucospilota} and \textit{Holothuria atra} specimens from the La Saline reef (mean ± SD).\textsuperscript{*}  

\begin{center}

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>$N$</th>
<th>TL (cm)</th>
<th>WM (g)</th>
<th>DW (g)</th>
<th>AFDW (g)</th>
<th>Cuvierian tubes time of expulsion (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{H. leucospilota}</td>
<td>TE</td>
<td>7</td>
<td>33.2 ± 5.7</td>
<td>355.6 ± 95.0</td>
<td>14.8 ± 5.6</td>
<td>10.0 ± 4.2</td>
<td>19.6 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>7</td>
<td>31.3 ± 8.1</td>
<td>458.3 ± 110.4</td>
<td>14.7 ± 2.8</td>
<td>9.8 ± 1.6</td>
<td>128.4 ± 128.1</td>
</tr>
<tr>
<td></td>
<td>CM</td>
<td>7</td>
<td>32.7 ± 6.5</td>
<td>430.6 ± 91.7</td>
<td>13.8 ± 4.1</td>
<td>9.6 ± 2.7</td>
<td>76.0 ± 67.8</td>
</tr>
<tr>
<td>\textit{H. atra}</td>
<td>TE</td>
<td>5</td>
<td>29.8 ± 7.3</td>
<td>319.4 ± 115.1</td>
<td>13.7 ± 4.9</td>
<td>9.3 ± 3.5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>7</td>
<td>14.1 ± 2.8</td>
<td>83.3 ± 34.1</td>
<td>3.2 ± 1.7</td>
<td>1.9 ± 1.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>CM</td>
<td>7</td>
<td>17.2 ± 4.7</td>
<td>116.7 ± 28.0</td>
<td>4.0 ± 1.4</td>
<td>2.4 ± 0.8</td>
<td>—</td>
</tr>
</tbody>
</table>

\textsuperscript{*}N number of samples, TL total length, WM wet mass, DM dry mass, AFDW ash-free dry weight. Data are reported as mean ± SD.
between AFDW and microsomal protein content of any tissue investigated for AChE and EROD activity measurements ($P < 0.05$ in each case). Several studies on marine organisms have noted the influence of growth on biomarker activity. Zinckl et al.\[49\] have shown that AChE activity was lower in older fish than in younger fish and such an influence was also highlighted for EROD activity.\[45\] No significant relationships were observed between AFDW and AChE activity levels in muscle and tentacle tegument tissues neither in Holothuria leucospilota nor in Holothuria atra (Table 4). EROD activity levels were poorly correlated to AFDW in both species with the exception of the tegument tissue in H. atra where activity levels increased with biomass (Table 4).

The dendrogram and MDS ordination (Fig. 5) performed on both AChE and EROD activity levels showed distinct sub-groupings in Holothuria leucospilota. Individuals from TE site are separated from individuals collected at PA and CM sites. ANCOVA analyses (with AFDW as covariate) revealed significant site effect on AChE activity levels measured in Holothuria leucospilota muscle tissue only (Table 2). Individuals from TE site exhibited lower activity levels (mean of $29.4 \pm 7.2 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$) compared to those sampled at PA and CM sites ($54.8 \pm 8.5$ and $60.7 \pm 7.2$).

EROD activity levels measured in Holothuria leucospilota did not significantly vary with location in any tissue considered (Table 2). Multivariate analysis also detected sub-groupings in Holothuria atra (Fig. 5) with individuals from TE and CM sites showing similar AChE and EROD responses, which were distinctly different from those collected at PA. However, there were no significant differences in AChE and EROD activity levels between Holothuria atra individuals sampled at TE, PA and CM regarding any tissue (ANCOVAs, Table 2): spatial grouping found in combined AChE and EROD responses were not detected in Holothuria atra when biomass was used as a cofactor. Therefore, including biomass in spatial analysis restricted conclusions from multivariate analyses for Holothuria atra, suggesting an effect of animal body condition. However, biomass patterns described previously did not explain spatial variability found in Holothuria atra activity levels, and we can not conclude to its significant influence. Since there is no evidence of a relationship between holothurian size/biomass and age (unexplored methods for age assessment in wild populations), this latter parameter may act as a possible confounding factor, and as such would require further investigation.

Nonetheless, AChE activity did not show any significant change in relation to biomass of the animals of both holothurian species, and this contributes to increasing the
Insecticide and herbicide molecules are chronically detected in Reunion Island river waters (e.g., diuron and atrazine desethyl max. 0.04 µg/L. Water Office of Reunion database, http://banquededonnees.eaureunion.fr). In further studies, toxicological survey should focus on anticholinesterase compounds concentration in reef sediments and waters to test whether the AChE activity level decrease in *H. leucospilota* at TE site is caused by their presence.

### Conclusion

This work consisted of a preliminary *in situ* approach for the improvement of AChE and EROD biomarkers in holothurians. Data obtained provide base-lines to estimate possible impact of Chikungunya arbovirus vector controls utilization in Reunion Island. Results of this study indicate that longitudinal body muscle and digestive tract wall are suitable tissues for measuring AChE and EROD activity respectively in both holothurian species. Effect of biometric parameter on EROD activity levels are highlighted for *H. atra* and may presume possible influence of age. One of the most important challenges in further research will be to characterize holothurian age, nutrition sources and the reproductive cycle in natural environment, and to test their effects on AChE and EROD activities. Since most marine animals express circa-annual variations in their basic physiology and biochemistry, seasonal variations in AChE and EROD activity should be further assessed.

### Acknowledgments

This research was supported by the French Conseil Régional de La Réunion. J.K. also benefited from support of the Conseil Régional de La Réunion and the European Social Fund through a PhD grant. The authors would like to thank Alexandre Cérou for sampling assistance. Prof P. Vasseur is thanked for helpful suggestions on a draft of the manuscript, and Drs K. Rogers and J.C. Russell for English corrections.

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