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Caribbean yellow band disease compromises the activity of catalase and glutathione S-transferase in the reef-building coral *Orbicella faveolata* exposed to anthracene

Luis Miguel Montilla[1,*], Ruth Ramos[2], Elia García[2], Aldo Cróquer[1]

[1]Universidad Simón Bolívar, Departamento de Estudios Ambientales, Laboratorio de Ecología Experimental, Apdo. 89000, Caracas, Venezuela

[2]Departamento de Biología de Organismos, Laboratorio de Comunidades Marinas. Apdo. 89000, Caracas, Venezuela

ABSTRACT: Healthy and diseased corals are threatened by different anthropogenic sources, such as pollution, a problem expected to become more severe in the near future. Despite the fact that coastal pollution and coral diseases might represent a serious threat to coral reef health, there is a paucity of controlled experiments showing whether the response of diseased and healthy corals to xenobiotics differs. In this study, we exposed healthy and Caribbean yellow band disease (CYBD)-affected *Orbicella faveolata* colonies to 3 sublethal concentrations of anthracene to test if enzymatic responses to this hydrocarbon were compromised in CYBD-affected tissues. For this, a 2-factorial fully orthogonal design was used in a controlled laboratory bioassay, using tissue condition (2 levels: apparently healthy and diseased) and pollutant concentration (4 levels: experimental control, 10, 30 and 100 ppb concentration) as fixed factors. A permutation-based ANOVA (PERMANOVA) was used to test the effects of condition and concentration on the specific activity of 3 enzymatic biomarkers: catalase, glutathione S-transferase, and glutathione peroxidase. We found a significant interaction between the concentration of anthracene and the colony condition for catalase (Pseudo-$F = 3.84$, df = 3, $p < 0.05$) and glutathione S-transferase (Pseudo-$F = 3.29$, df = 3, $p < 0.05$). Moreover, our results indicated that the enzymatic response to anthracene in CYBD-affected tissues was compromised, as the activity of these enzymes decreased 3- to 4-fold compared to healthy tissues. These results suggest that under a potential scenario of increasing hydrocarbon coastal pollution, colonies of *O. faveolata* affected with CYBD might become more vulnerable to the deleterious effects of chemical pollution.

KEY WORDS: Caribbean yellow band disease · *Orbicella faveolata* · Enzymatic biomarkers · Anthracene · Catalase · Glutathione S-transferase

INTRODUCTION

Coral reef ecosystems are highly susceptible to anthropogenic impacts (Hughes et al. 2003), as their distribution often overlaps with densely populated coastal areas (Mora 2008), and they are thus exposed to pollutants such as polycyclic aromatic hydrocarbons (PAHs), heavy metals and pesticides. The chronic input of these xenobiotic compounds compromises water quality (Shen et al. 2013), challenging the resilience of these ecosystems as corals are stressed by bleaching and emergent diseases (Hughes et al. 2003, Burge et al. 2014). There is overwhelming evidence that the increasing prevalence and incidence of coral diseases is one of the factors contributing to the accelerated degradation of modern reefs (Weil 2004, Ruiz-Moreno et al. 2012). Moreover, the rapid emergence and the deleterious effects of these diseases have

*Corresponding author: luismmontilla@usb.ve

often been correlated with a combination of global (Burge et al. 2014) and local threats, particularly important ones being eutrophication (Bruno et al. 2003, Voss & Richardson 2006), thermal stress (Bruno et al. 2007) and pollution (Kaczmarsky et al. 2005).

Corals are naturally exposed to xenobiotic compounds, such as metabolites produced by competing species (Pawlik et al. 2007, Chaves-Fonnegra et al. 2008, Rasher & Hay 2010, Rasher et al. 2011), and also to oxidative stress since their habitats receive a high incidence of UV irradiance. Nevertheless, they are able to cope with these stressors because they possess a series of enzymatic mechanisms. These include antioxidant activity and a series of biotransformation pathways involving the addition of more polar groups to the xenobiotic (i.e. Phase I) and the conjugation of this intermediate compound with other substrates (i.e. Phase II; Downs et al. 2012, Rougée et al. 2014).

These enzymatic pathways are not only activated under natural conditions, but also in the presence of anthropogenic xenobiotics. For this reason these biomarkers have proven to be a valuable tool in environmental monitoring programs as early sentinels of stress for a myriad of organisms, including corals (Downs et al. 2000, 2005, 2011). For example, the exposure to PAHs leads to a higher expression of antioxidant and xenobiotic-metabolizing enzymes in healthy corals (Ramos & García 2007). These markers have also been utilized to detect specific responses of corals to thermal stress, diseases (Palmer et al. 2011a) and pollutants such as heavy metals and antifouling compounds (Downs & Downs 2007, Venn et al. 2009). Thus, the use of these enzymatic biomarkers as proxies of coral health is increasing as new biomarkers and methodologies are being developed (Venn et al. 2009, Mydlarz & Palmer 2011, Ramos et al. 2011, Downs et al. 2012, Anithajothi et al. 2014, Rougée et al. 2014).

Among the many enzymes described for corals, glutathione S-transferase (GST), catalase (CAT) and glutathione peroxidase (GPX) are commonly used, as they are present in virtually all marine organisms (Livingstone 1991, Rewitz et al. 2006). GST plays a central role in the Phase II biotransformation of intermediary metabolites formed during exposure to xenobiotics by conjugating these products with glutathione. Furthermore, GST participates in the elimination of hydroperoxides, thereby overlapping with the activity of GPX (Hayes & McLellan 1999, Limón-Pacheco & Gonsebatt 2009). CAT destroys reactive oxygen species (ROS) formed by thermal stress or the activity of other antixenobiotic enzymes. Thus, these enzymes are very important as they protect corals from the deleterious effects of different stressors. While different studies have shown that healthy corals increase the activity of these enzymes when exposed to pollutants (Venn et al. 2009, Gust et al. 2014), no studies have determined whether diseased corals fail to activate these mechanisms under controlled exposure to xenobiotic compounds.

Caribbean yellow band disease (CYBD) is a widespread coral syndrome affecting several coral species, usually of the genera *Orbicella* and *Montastraea*. This disease produces rapid and extensive mortality of living tissues of its hosts, but more importantly, reduces fecundity (Weil et al. 2009), causes structural damage and energetically compromises the zooxanthellae (Cervino et al. 2001, Morgan et al. 2015). It also alters the gross composition of tissues by decreasing the ratio of proteins and carbohydrates in relation to the amount of lipids in CYBD lesions (Guerra et al. 2014). The etiology of CYBD has been associated with 3 different strains of *Vibrio* (Cervino et al. 2008, Weil et al. 2008).

At microbial scales, not only the structure of the bacterial communities associated with the holobiont (Cróquer et al. 2012), but also their function, have been shown to differ between healthy and CYBD-affected tissues (Kimes et al. 2010). Recent studies found that the immune response in CYBD-affected *Orbicella faveolata* is altered and diminished in comparison with healthy colonies after a bleaching event (Mydlarz et al. 2009). Given the series of deleterious effects that CYBD produces on its coral hosts, it should be not surprising that this syndrome also affects the mechanisms of defense of corals exposed to xenobiotic compounds.

Among many xenobiotics, anthracene is one of the most common compounds found in polluted coastal marine environments. It is one of the PAHs prioritized by the US EPA and often occurs in Venezuelan coastal waters (Garcia 2014). Anthracene has been frequently used in toxicological studies because of its bioaccumulative potential and photoenhanced toxicity and has been tested in a wide range of marine organisms. Its effects include reducing survival and fecundity in planktonic organisms (Allred & Giesy 1985, Holst & Giesy 1989, Bi et al. 2015), lower growth rates in algae (Gala & Giesy 1992), neurotoxicity and oxidative stress in fishes (Palanikumar et al. 2012), and it is usually assessed in specimens of commercial interest that may bioaccumulate this compound (Perugini et al. 2007).

While recent studies have provided invaluable information showing that CYBD has significant im-

pacts on important physiological and immunological processes in the host under natural conditions and/or after abnormal periods of thermal stress, there is a paucity of data from manipulative and controlled experiments comparing anti-xenobiotic responses in diseased corals. We hypothesized that enzymatic activity of CYBD-affected tissues exposed to anthracene would be altered as a consequence of the coral disease. In order to test this hypothesis, we performed a controlled laboratory experiment using 3 sublethal concentrations of anthracene on apparently healthy and CYBD-affected fragments of *O. faveolata*.

MATERIALS AND METHODS

Study site and sample collection

Samples were taken from Cayo Sombrero (Venezuela) where CYBD is highly prevalent (4–5%) in the coral community and primarily affects *Orbicella annularis* and *O. faveolata* populations (Cróquer & Bone 2003). Nine fragments from 4 apparently healthy (without any visible sign of the disease) and 4 CYBD-affected *O. faveolata* colonies were collected using chisel and hammer (n = 36 apparently healthy + 36 CYBD-affected = 72 fragments). The diseased fragments were collected including the transition border between the yellow band and the healthy tissue. Apparently healthy fragments from CYBD-affected colonies were not considered in this study because our goal was to test if CYBD compromised the response to the xenobiotic in affected tissues and not if these effects were systemic. The fragments were transported individually to the surface in resealable plastic bags and then taken to the laboratory in one container for apparently healthy fragments and in a separate container for diseased fragments, both filled with seawater.

Experimental design and bioassay

A 2-factor and fully orthogonal experimental design was used to test the effect of tissue condition (fixed factor with 2 levels: healthy and diseased), anthracene concentration (fixed factor with 4 levels: experimental control, 10, 30 and 100 ppb) and the interaction between these 2 factors. Each concentration was replicated in 3 glass aquaria containing 3 coral fragments each for both tissue conditions (24 aquaria). The aquaria were oxygenated with a bubbling system and illuminated with 40 W fluorescent bulbs on a 12 h light:12 h dark daily cycle.

Anthracene concentrations in the aquaria were prepared by pipetting 97, 300 and 970 µl of a stock solution of anthracene (552 ppm dissolved in pure ethanol) in a total volume of 5.5 l seawater (for 10, 30 and 100 ppb, respectively) in each aquarium. These concentrations were chosen based on technical reports on concentrations of PAHs in Venezuelan coastal waters where anthracene has been detected in concentrations up to 200 ppb (Garcia 2014). We did not use a procedural control for ethanol because the proportion of this solvent in relation to the water in the aquaria was 220 times smaller than anthracene and because of the high volatility of ethanol in relation to anthracene. Additionally, the use of a procedural control to test the effect of ethanol would have required the sacrifice of more healthy colonies. In other toxicological assays, using ethanol does not appear to have an effect on the coral response (Hasue et al. 2013); however, we are aware that the response of the coral to anthracene in this experiment could be caused by a combined effect of anthracene and the ethanol.

The samples were maintained in the aquaria for 48 h in seawater brought to the lab from the study site for acclimatization prior to anthracene exposure. In the first hours, there was mucus release in several colonies from both conditions. We made changes in the seawater from all the aquaria until we observed that the water remained clear and without signs of mucus residuals. After acclimatization, a dose of anthracene was administered every 24 h for 96 h. During the experiment (and the acclimatization period), salinity and dissolved oxygen concentration averaged 37.9 ± 1.6‰ and 7.65 ± 0.5 ppm, respectively. After 96 h, each fragment was immediately frozen using liquid nitrogen and stored at −80°C to determine the activity of CAT, GST and GPX in coral tissues.

Tissue extraction

All coral fragments were rinsed with phosphate buffer (pH = 7.6, 100 mM) containing sucrose (125 mM), β-mercaptoethanol (5 mM) and the following protease inhibitors: EDTA (1 mM), iodoacetamine (0.1 mM) and phenylmethanesulfonyl fluoride (1 mM). The coral tissue was removed from the skeleton with compressed air, and then the tissue-buffer slurry was homogenized at 2500 rpm with a Potter-Elvehjem polytetrafluoroethylene pestle for 30 s. The homogenate was centrifuged twice, first at 200 × *g* for 6 min at 4°C and then at 10 000 × *g* for 25 min at

4°C. The remaining supernatant was used for the estimation of the activity of enzymatic biomarkers.

Activity of enzymes in response to anthracene

The activities of CAT, GST and GPX were determined following the methods outlined by Aebi (1984), Habig et al. (1974) and Zakowski & Tappel (1978), and (Wendel 1980), respectively. Briefly, for CAT we added 100 µl of the supernatant to a solution of 2900 µl of phosphate buffer (pH = 7.0, 80 mM) and 30 mM of H_2O_2 and measured the absorbance at 240 nm for 1 min. For GST, we added 150 µl of the supernatant to 850 µl of a solution of phosphate buffer (pH = 6.5, 1 M), reduced glutathione-S (GSH) 100 mM and 1-Cl-2,4-dinitrobenzene (DNTB) 100 mM. Then we measured the absorbance at 340 nm for 3 min. The activity of GPX was estimated by direct determination of the GSH content. In this case we incubated 500 µl of the supernatant with a mixture of 200 µl of phosphate buffer (0.4 M; pH = 7), 200 µl GSH (10 mM), 100 µl sodium azide (10 mM) and 100 µl H_2O_2 (5 mM) at 37°C for 10 min. After this, we added 400 µl of trichloroacetic acid (TCA) (10%) and centrifuged the mixture at 3200 × *g*. Finally, we added 500 µl DNTB (10 mM) to the supernatant and measured the absorbance (*A*) at 340 nm for 3 min. Given the non-linear nature of this reaction, the activity was estimated as activity = $\ln(\Delta A_{340})$/time, sensu Zakowski & Tappel (1978). All the enzymatic activities were standardized by the total protein content of each sample, which was estimated using the Bradford method (Bradford 1976).

Data analysis

Differences in the enzymatic activity were tested using an univariate permutation-based ANOVA (PERMANOVA, Anderson 2001) based on a matrix of Euclidean distances with no data transformations. This test allowed us to perform an ANOVA without needing to transform the data to achieve the normality. However, as this test is sensitive to the dispersion of data, which could lead to an increased probability of hypotheses rejection (Anderson & Walsh 2013), we performed a test of homogeneity of dispersions (PERMDISP) to verify whether the dispersion of data for the 3 variables would hamper our conclusions. CAT and GPX did not have a statistically significant dispersion of the data whereas GST did. This dispersion in the variance actually coincided with the 2 levels where we found a significant effect of the concentrations of anthracene, so rather than labeling this as a dispersion problem, it can be indicative of an effect not only in the mean activity but also in its variance as well.

Pairwise post hoc analyses based on permutations were performed only for significant sources of variation. The analyses were performed with PRIMER (Clarke & Gorley 2006). We also estimated the power of the test using G*Power (Faul et al. 2007) for those cases when the null hypotheses was not rejected.

RESULTS

The results indicated that the optimum response for apparently healthy tissues exposed to anthracene occurred at 30 ppb, this pattern being consistent for the 3 enzymes tested (Figs. 1, 2 & 3). Furthermore, the effect of anthracene on the response of CAT and GST was statistically different depending simultaneously on the concentrations used and the condition of the tissue. For GPX we did not find statistical differences for any of the sources of variation (Table 1).

In apparently healthy tissues, the specific activity of CAT increased by 1.5 to 2-fold at 30 ppb compared to the control and 100 ppb (Pseudo-F = 3.8412; p = 0.017, Table 1). The opposite trend was observed for CYBD tissues, which had a lower or equal activity

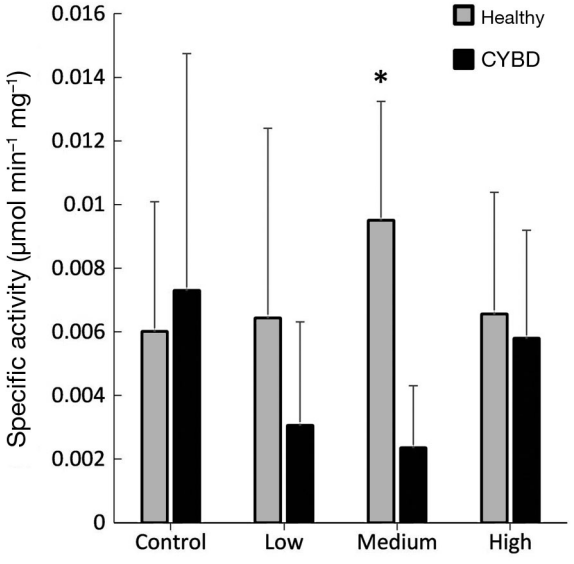

Fig. 1. Mean specific activity of catalase in healthy and Caribbean yellow band disease (CYBD)-affected corals exposed to 4 anthracene concentrations. (*) Differences between the conditions with p < 0.05 for pairwise comparisons. Error bars are SD

compared to their controls, this pattern being consistent for all concentrations. Pairwise comparisons for the interaction indicated that the mean specific activity was statistically different only for the medium concentration (t = 5.489, p = 0.001, Fig. 1). Furthermore, the mean activity measured on CYBD tissues was 75% lower compared with the activity recorded in apparently healthy ones only at 30 ppb (Fig. 1).

Similarly, the activity of the GST was also statistically different for the interaction between the condition of tissues and the concentration (Pseudo-F = 3.2993, p = 0.024). For this enzyme, the pattern

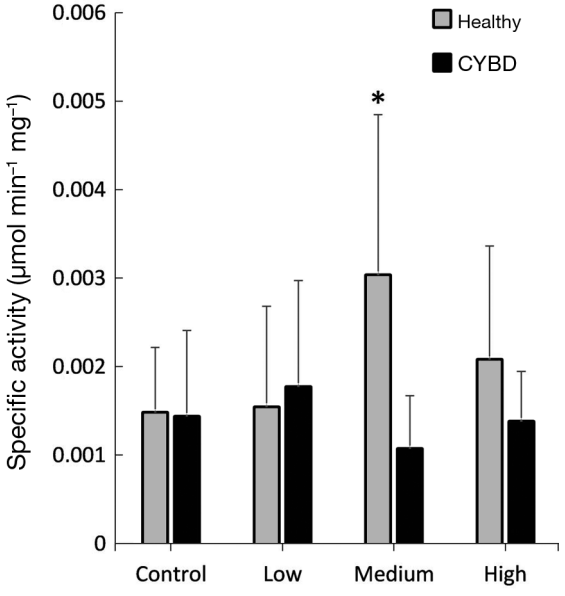

Fig. 2. Mean specific activity of glutathione S-transferase in healthy and Caribbean yellow band disease (CYBD)-affected corals exposed to 4 anthracene concentrations. (*) Differences between the conditions with p < 0.05 for pairwise comparisons. Error bars are SD

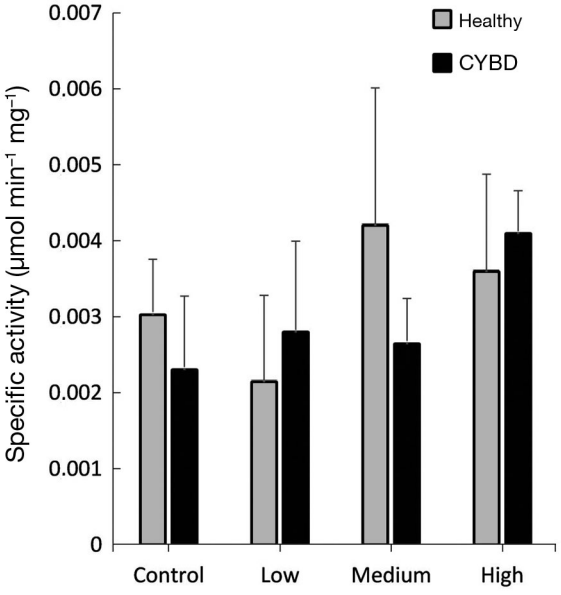

Fig. 3. Specific mean activity of glutathione peroxidase in healthy and Caribbean yellow band disease (CYBD)-affected corals exposed to 4 anthracene concentrations. Error bars are SD

Table 1. Permutation-based univariate analysis of variance using Euclidean distances of specific activity of catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPX)

	df	SS	MS	Pseudo-F	p(perm)	Unique perms
Specific activity of CAT						
Condition	1	1.99×10^{-4}	1.99×10^{-4}	10.06	0.002	9816
Concentration	3	6.25×10^{-5}	2.08×10^{-5}	1.03	0.37	9958
Condition × Concentration	3	3.16×10^{-4}	1.05×10^{-4}	5.31	0.002	9963
Residuals	121	2.40×10^{-3}	1.99×10^{-5}			
Total	128	2.97×10^{-3}				
Specific activity of GST						
Condition	1	4.18×10^{-7}	4.18×10^{-7}	0.16	0.68	9813
Concentration	3	8.94×10^{-6}	2.98×10^{-6}	1.14	0.34	9958
Condition × Concentration	3	3.30×10^{-5}	1.10×10^{-5}	4.21	0.008	9949
Residuals	130	3.39×10^{-4}	2.61×10^{-6}			
Total	137	3.81×10^{-4}				
Specific activity of GPX						
Condition	1	3.81×10^{-2}	3.81×10^{-2}	0.57	0.46	9830
Concentration	3	2.31×10^{-1}	7.71×10^{-2}	11.46	0.33	9958
Condition × Concentration	3	2.57×10^{-1}	8.57×10^{-2}	12.74	0.29	9950
Residuals	73	4.91×10	6.73×10^{-2}			
Total	80	5.48×10				

recorded in apparently healthy tissues was similar to the one observed for CAT, with the mean specific activity increasing by 1.5 to 2.8-fold for the medium concentration compared to all other treatments (Fig. 2). We found a significant reduction of 64% in the activity of GST in CYBD compared to apparently healthy tissues ($t = 3.084$, $p = 0.008$, Fig. 2). Thus, the results indicate that the optimum response of CAT and the GST to anthracene in apparently healthy tissues occurred at 30 ppb, whereas in CYBD tissues this response was seriously compromised.

Opposite to CAT and GST, the specific activity of GPX was not statistically significant either for the interaction (Pseudo-$F = 12.741$, $p = 0.75$) or the main effects for each treatment (Fig. 3). Nevertheless, the power of this test was 99% for an effect size of 71%, further suggesting that more replicates would be needed in order to detect differences given the high variability recorded among replicates.

DISCUSSION

Our results indicate that CYBD compromises antixenobiotic responses in the reef-building coral *Orbicella faveolata*. CYBD tissues failed to respond as apparently healthy tissues to increasing concentrations of anthracene. While the underlying mechanisms explaining this pattern remain unclear and must be further investigated, our controlled experiment showed that CYBD tissues might be more vulnerable to the exposure to PAHs such as anthracene compared to apparently healthy corals.

When exposed to PAHs, whether from water or sediments, healthy corals bioaccumulate these xenobiotics (Peters et al. 1981, Knap et al. 1982, Solbakken et al. 1984, Ko et al. 2014). In cases of chronic exposure, a series of sublethal effects can include lower fecundity (Rinkevich & Loya 1979, Peters et al. 1981) accompanied by loss of zooxanthellae, an increase in the size and number of mucus secretory cells and overall atrophy and fragmentation of cells (Peters et al. 1981). In the long term, the exposure can lead to death of coral colonies and a concomitant loss of live cover and eventually the flattening of the tridimensional structure of the reef (Bak 1987).

As part of the detoxification response, healthy corals increase the activity of antioxidant and antixenobiotic enzymes such as CAT and GST when exposed to PAHs (Downs et al. 2006, Rougée et al. 2006, Ramos & García 2007). Our results showed that optimum response to anthracene in apparently healthy tissues occurs at 30 ppb; nevertheless, the response was variable among coral fragments, further illustrating that individual coral colonies may respond quite differently to stress. Our results are consistent with other studies showing that the enzymatic responses of healthy organisms are highly variable depending on species, time of exposure and the concentration and type of xenobiotic (Bosveld et al. 2002, Downs et al. 2006, Rougée et al. 2006, Downs & Downs 2007, Guzmán-Martínez et al. 2007, Ramos & García 2007, Ramos et al. 2011).

The lower activities of CAT and GST at the highest concentrations recorded on apparently healthy tissues suggest that the enzymatic defense mechanisms of *O. faveolata* may be losing effectiveness at higher concentrations of anthracene. This decay in the activity of antixenobiotic enzymes and other immune functions in presence of PAHs has been reported in other marine organisms (Grundy et al. 1996, Le Moullac & Haffner 2000, Hylland 2006). A possible explanation is that the highest concentrations of anthracene utilized in our experiment surpassed the threshold beyond which these enzymes are not expressed, as reported for other scleractinian corals exposed to several pollutants (Morgan & Snell 2006). In this case, this threshold may be established by the depletion of the glutathione as seen in other organisms in presence of PAHs (Hannam et al. 2010). Without glutathione, the GST cannot conjugate the intermediate metabolites for its excretion. Alternatively, the prolonged toxicity (96 h of exposure) may have produced structural damage to the coral cells thereby disabling them for synthesizing CAT and GST.

Opportunistic infections and other health problems represent an additional challenge for corals exposed and/or vulnerable to pollution. The compromise of immunity has been extensively reported in diseased corals (Mydlarz et al. 2009, Palmer et al. 2011b, Pinzón et al. 2015). Particularly, CYBD is known to produce a myriad of deleterious effects on coral hosts and their microbial partners, encompassing structural damages to the zooxanthellae (Cervino et al. 2001, 2004) and significant changes in the structure and function of these microbial associates (Kimes et al. 2010, Cróquer et al. 2012, Closek et al. 2014). The stability of these associations plays an important role in coral health (Mouchka et al. 2010); therefore, a permanent or a transitory impairment of these relationships might also disrupt the mechanisms of defense of corals to xenobiotic compounds. For example, it is widely accepted that zooxanthellae play an important role in the detoxification and bioaccumulation process, in some cases exhibiting higher activity levels for some antioxidant, antixenobiotic

enzymes and higher concentration of accumulated compounds compared to the coral polyp (Kennedy et al. 1992, Gust et al. 2014). In other experiments, the exposure of *Porites divaricata* to fluoranthene (another PAH similar to anthracene) at concentrations similar to the ones we used led to a diminished photosynthetic efficiency and eventually to bleaching in branches exposed to sunlight (Guzmán-Martínez et al. 2007). Thus, it is conceivable that anthracene could be inducing damage to the zooxanthellae associated with healthy and diseased *O. faveolata,* compromising the enzymatic responses or reducing its synthesis in both healthy and CYBD-affected tissues exposed to high doses of this PAH. Such compromise is worse in CYBD-affected tissues because of the additional stress this pathology imposes not only for the zooxanthellae, but also for the coral holobiont (Mydlarz et al. 2009, Weil et al. 2009, Guerra et al. 2014, Morgan et al. 2015). This hypothesis, along with the underlying cellular mechanisms involved, remains to be formally tested.

In conclusion, we found that the ability to cope with and/or defend against chemical pollution is compromised or diminished in CYBD-affected tissues. These results suggest that CYBD corals might become more vulnerable to chronic pollution problems. With CYBD highly prevalent across the Caribbean region, primarily affecting the genus *Orbicella*, and with human impacts increasing in frequency and intensity, this disease is a serious hazard for Caribbean corals in the short term.

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