Journal Pre-proof

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PII: S0013-9351(20)30397-2

DOI: https://doi.org/10.1016/j.envres.2020.109504

Reference: YENRS 109504

To appear in: *Environmental Research*

Received Date: 6 January 2020

Revised Date: 6 April 2020

Accepted Date: 7 April 2020

Please cite this article as: Campos, P., Pires, Adí., Figueira, E., Can *Palythoa* cf. *variabilis* biochemical patterns be used to predict coral reef conservation state in Todos Os Santos Bay?, *Environmental Research* (2020), doi: https://doi.org/10.1016/j.envres.2020.109504.

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Can *Palythoa* cf. *variabilis* biochemical patterns be used to predict coral reef conservation state in Todos Os Santos Bay?

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Abstract

Coral reefs are one of the most diverse, complex and productive marine ecosystems on the planet. Global climate change and other anthropogenic impacts have had a strong impact on the equilibrium of these ecosystems and causing the denominated “coral reef crisis”. One consequence of coral reef crisis is the phase shift in reef communities, where scleractinian corals responsible for the bioconstruction of the coralline building are replaced by macroalgae or soft corals. In Todos os Santos Bay (TSB) there is a rare case of phase shift caused by the soft coral *Palythoa* cf. *variabilis*. When in population outbreak, this coral species becomes dominant and leads to loss of scleractinian coral cover. *Palythoa* genus establishes a symbiotic relationship with dinoflagellate algae of the genus *Symbiodinium*, that is changed in phase shift coral reefs, but other alterations remain unknown. In this study, the metabolism (oxidative damage, antioxidant and biotransformation enzymes, electron transport chain activity and photosynthetic pigments) of *P*. cf. *variabilis* from reefs in different conservation states was studied to identify and relate if changes that may occur in the biochemical and metabolism of the coral might trigger the population outbreak, identify parameters recognizing if corals are in stress and assess if one or more parameters can reflect the level of stress organisms are experiencing. The results obtained evidenced a clear distinction in the biochemistry and metabolism of corals from conserved sites and sites in phase shift, and these changes may be the trigger for population outbreak. Some of the parameters were able to discriminate the level of stress corals are experiencing and may allow to recognize the most at-risk coral reefs that need immediate intervention and prevent the entry into or revert *P. cf. variabilis* outbreak and phase shift in coral reefs. Actions like these can be of vital importance for the preservation of TSB coral reefs and possibly for other threatened reefs worldwide.

Keywords: Soft coral, oxidative stress, Phase shift

1. Introduction

Coral reefs provide refuge, spawning, rearing, feeding and breeding too many species, and are an important source of food and economic resources for coastal populations (Moberg and Folke, 1999). Therefore, they are one of the most diverse, complex and productive marine ecosystems on the planet, generating goods and services for all humankind.

Small changes on environmental conditions, such as temperature increase, have been shown to impact the equilibrium of these ecosystems strongly, highlighting their high vulnerability (Brown et al., 2002; Downs et al., 2002, 2013; Peterson et al., 2018). Global climate change and other anthropogenic impacts have been causing the 'coral reef crisis' (Bellwood et al., 2004, Carreón-Palau et al., 2017, Petersen et al., 2018), with estimates of 19% of total loss and 35% of threatened areas worldwide (Aronson et al., 2003; Bellwood et al., 2004; Hughes et al., 2017; Wilkinson, 2008). One consequence of the coral reef crisis is the phase shift in reef communities, where scleractinian corals responsible for the bioconstruction of the coralline building are replaced by other organisms (Cruz et al., 2014). This phenomenon has been extensively studied when it comes to macroalgal dominance (Bruno et al., 2009; Fung et al., 2011; Knowlton, 1992; McCook, 1999; McManus and Polsenberg, 2004; Norström et al., 2009; Nyström et al., 2000; Nyström and Folke, 2001), but there is a gap when this dominance refers to other organisms (Norström et al., 2009).

Todos os Santos Bay (TSB) is the second-largest bay in Brazil (1235 km^2). This coastal system is close to Salvador city, which has an estimated population of 2.8 million in 2018 (IBGE, WEB, 2019). Over the past 50 years, reefs have been severely degraded by anthropogenic impacts (Beretta et al., 2014; Celino et al., 2012; Dutra and Haworth, 2008; Milazo et al., 2016; Roth et al., 2016). Santos (2016) and Hatje et al. (2009) reported differences in contamination by metals and PAHs in the central zone of TSB, with the area near Maré island presenting higher contamination, especially by Cu, Pb, Ni, Cr and Zn and PAHs, and the area near Frades island the lowest concentrations of metals detected (Santos 2016). Celino et al. (2012) also showed low contamination by PAHs in the water near Frades island, while Hatje et al. (2009) reported contamination by PAHs in the area near Maré island. Most of the areas in the present study are precisely located between these two islands. Thus, the sites near Frades island would be subjected to a lower level of contamination, while the sites near Maré island would be influenced by the water coming from Aratu bay and will be exposed to higher contamination levels, dominated by

chemical, metal-mechanic and metallurgical industry, implanted in the surrounding area of this bay (Souza, 2014; Santos 2016).

In TSB there is a rare case of phase shift caused by the soft coral *Palythoa* cf. *variabilis* (Figure 1A and 1B). When in population outbreak, this species causes an ecological imbalance and leads to loss of scleractinian coral cover by competition with *Palythoa* cf. *variabilis* that becomes the dominant coral species, reducing biodiversity, trophic structure and local ecological functions (Cruz, et al., 2015a; Cruz et al., 2015b; Cruz et al., 2016). *Palythoa* genus establishes a symbiotic relationship with dinoflagellate algae of the genus *Symbiodinium* (Davy et al., 2012). This symbiosis provides much of the energy and oxygen required for coral feeding and respiration through photoassimilates and oxygen (Davy et al., 2012). In return, the coral provides carbon dioxide, nitrogenous compounds and phosphorus to the dinoflagellate (Davy et al., 2012; Falkowski et al., 1984). Under suboptimal conditions, such as stress, the symbiosis is affected (Davy et al., 2012) and growth, photosynthesis and nutrient exchange are changed (Wooldridge, 2013). Thus, it is surprising why metabolic alterations and oxidative damage in corals from threatened reefs are so poorly known.

When facing a challenge, organisms adjust their metabolism (protein content) in order to adapt to the changing conditions and to minimize damage, such as triggering mechanisms to restrain oxidative stress (Pires et al., 2017; Sokolova, 2013). Antioxidant enzymes (e.g. superoxide dismutase, glutathione peroxidase and catalase) have the ability to scavenge reactive oxygen species (ROS), mitigating oxidative stress (Regoli and Giuliani, 2014). Biotransformation enzymes (e.g. glutathione S-transferases) detoxify cells from reactive xenobiotic metabolites of exogenous (e.g. polycyclic aromatic hydrocarbons) and endogenous origin (e.g. lipid hydroperoxides) (Newman and Unger, 2003; Wright and Welbourn, 2002). When antioxidant and biotransformation responses are not able to decrease ROS to physiological levels, cell damage (lipid peroxidation and protein carbonylation) overcomes (Valavanidis et al., 2006).

Previous studies have shown changes in the number and photosynthetic ability of microsymbionts associated with these corals (Rabelo et al., 2014; Santos et al., 2016). However, alterations in cellular metabolism that may occur in *P.* cf. *variabilis* and that may underlie their dominance in phase shift coral reefs remain unclear.

The aim of this study was to:1) identify and relate the changes that may occur in the biochemistry and metabolism of *Palythoa* cf. *variabilis* that might trigger the population outbreak; 2) identify

parameters recognizing if corals are in stress; 3) assess if one or more parameters can reflect the level of stress organisms are experiencing.

2. Materials and methods

2.1. Description of the study area and field sampling

2.1.1. Description of the study area

Todos os Santos Bay (TSB), Brazil (12°50'S and 38°38'W) is the second-largest bay of Brazil, with an area of 1112 km^2 and an approximate maximum width of 32 km and 50 km in length, and an intertidal area of 327 km^2 (Hatje and Barros, 2012). The bay is inserted in a region with the highest biodiversity in the South Atlantic Ocean (Laborel, 1970), with ideal conditions for the development of coral reefs (Hatje et al., 2009; Santos, 2016). The water depths vary between 2 and 100 m, hot and humid tropical climate with an annual rainfall of around 2100 mm per year, with average water temperature around 25 °C (Santos, 2016). It is an Environmental Protection Area of the state of Bahia (Bahia, 1999), equivalent to category V, IUCN (International Union for Conservation of Nature) (Silva, 2005).

2.1.2 Sampling procedure

For this study *Palythoa* cf. *variabilis* was sampled from coral reefs at conserved (Figure 1A) and in phase shift (Figure 1B) in the eastern central part of TSB (Figure 1C), the region where most coral reefs within the bay are located (Dutra and Haworth, 2008) and a phase-shift coral reef near the Atlantic coast (Figure 1C).

Hatje et al. (2009) and Santos (2016) reported concentrations of metals (Cd, Cr, Cu, Ni, Pb and Zn), metalloid (As) and PAHs for the central eastern part of TSB (between Frades and Maré islands) lower than the northern part of the bay and below threshold effect level (TEL).

Palythoa cf. *variabilis* colonies were collected in April 2018 using SCUBA, transported to the laboratory in falcon tubes in ice and stored at -20ºC until transport. The colonies were transported in dry ice to the Biology Laboratory at Aveiro University, Portugal, where they were frozen at -80ºC. The stations were divided in phase shift reefs: R1, R2, R3, R4; and conserved reefs: R5, R6, R7 (Figure 1C) (Cruz et al., 2015a, Cruz et al., 2016).

2.2. Biochemical parameters

For biochemical analysis, frozen organisms were homogenized under liquid nitrogen and divided into subsamples. Extraction was performed with specific buffers (1:2, w/v) to determine: lipid peroxidation and protein carbonylation levels, the activity of antioxidant (superoxide dismutase, catalase, glutathione peroxidases) and biotransformation (glutathione S-transferases) enzymes, protein content and electron transport system activity. For lipid peroxidation (LPO), samples were extracted using 20% (v/v) trichloroacetic acid (TCA). For superoxide dismutase (SOD), catalase (CAT) glutathione peroxidases (GPx), glutathione S-transferases (GSTs) activity, protein content (PROT) and protein carbonylation (PC), potassium phosphate buffer (50mM potassium dihydrogen phosphate; 50mM dipotassium phosphate; 1mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1mM dithiothreitol (DTT), pH 7.0) was used. For ETS activity quantification, supernatants were extracted in 0.1 M Tris–HCl (pH 8.5), 15% (w/v) PVP, 153 mM magnesium sulfate (MgSO4) and 0.2% (v/v) Triton X-100. Samples were sonicated for 15s in ice and centrifuged at 4 °C for 20 min at 10000 g (or 3000 g for ETS). Absorbances were read in Biotek Synergy HT microplate reader, and Gen5 software was used for data collection.

2.2.1. Oxidative damage

LPO was measured by the quantification of thiobarbituric acid reactive substances (TBARS), following the methodology described by Buege and Aust (1978). This procedure is based on the reaction of LPO products, as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA), producing TBARS, that were quantified spectrophotometrically at 532 nm and calculated using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}). Results were expressed in nmol of MDA equivalents per g of fresh weight (FW).

Protein carbonylation (PC) was determined by the quantification of carbonyl groups (CG), according to the DNPH alkaline method (Mesquita et al., 2014) with some modifications (Udenigwe et al., 2016). The amount of CG was quantified spectrophotometrically at 450 nm ($22.308 mM^{-1}$ cm^{-1} extinction coefficient), and results were expressed in nmol of CG per g of FW.

2.2.2. Antioxidant and biotransformation enzymes

SOD activity was determined following the methodology described by Beauchamp and Fridovich (1971), that uses the reaction of nitro blue tetrazolium (NBT) with superoxide

radicals to form NBT diformazan. The absorbance was measured at 560 nm. SOD activity was expressed in units (U) per g FW, where U corresponds to the amount of enzyme that inhibited NBT diformazan formation by 50%.

CAT activity was determined following the methodology described by Johansson and Borg (1988). In this procedure, the enzyme reacts with methanol in the presence of hydrogen peroxide. The formaldehyde production was spectrophotometrically measured at 540 nm, with purpald as cromogen. Results were expressed in U per g FW, where U is defined as the amount of enzyme that caused the formation of 1.0 nmol formaldehyde, per min, under the assay conditions.

GPX activity was determined according to Paglia and Valentine (1967), where cumene hydroperoxide was used as substrate, using a glutathione reductase coupled assay to monitor the reduction of oxidized glutathione (GSSG). NADPH was added to measure the basal rate of GSSG reduction by monitoring the absorbance at 340 nm for 5 min (ε = 0. 00622 μM^{-1} cm^{-1}). GPx activity was expressed in U per g FW, where U is defined as the amount of enzyme that oxidized 1 nmol of NADPH per min.

The activity of GSTs was determined following the methodology described by Habig et al. (1974), adapted to microplate. The absorbance was measured at 340 nm. The activity was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. Results were expressed in U per g FW, where one unit (U) of enzyme was defined as the amount of enzyme that caused the formation of 1 µmol of thioeter per min under the assay conditions.

2.2.3. Metabolism parameters

For protein content (PROT), the extraction was performed with potassium phosphate buffer. Total PROT content was determined by the Biuret method, following the method of Robinson and Hogden (1940), and using bovine sorum albumin (BSA) as standard. The colourimetric reaction was carried out at room temperature for 10 min, and absorbance was measured at 540 nm. Results were expressed in mg per g FW.

The Electron Transport System (ETS) activity was measured according to King and Packard (1975) methodology with modifications (Coen and Janssen, 1997). Absorbance was measured in a microplate reader at 490 nm every 25 seconds for 5 minutes. The amount of formazan formed was calculated using ε = 15.900 M^{-1} cm^{-1} and the results expressed as nmol per minute per g FW.

2.3. Photosynthetic pigments

2.3.1. Extraction

The extraction of photosynthetic pigments from dinoflagellates was adapted from Jeffrey and Haxo (1968). Samples (0.3 g) were homogenized in the dark with 2 mL of 90% acetone using a mortar and pestle and centrifuged for 2 min at 3000 g and 4 °C. After centrifugation, the supernatant was passed to a new tube, and the pellet resuspended in 2 mL of 90% acetone and centrifuged again. The extraction procedure was repeated until the pellet had a greyish-white colour. In the end, acetone supernatants were combined.

2.3.1. Determination of pigments.

Chlorophylls a and c (chl a and chl c) and peridinin present in the extracts were determined spectrophotometrically, by determining the absorbance at 469, 663, 630 and 750 nm. Chlorophylls concentration were calculated using the equations described by Jeffrey and Haxo (1968), chl a = 13.31*A630 - 0.27*A663 and chl c = -8.37*A663+ 51.72*A630. Peridinin was calculated using the absorbance at 469 nm and $\text{Ɛ}_{1\%}$= 1330 cm^{-1}. Results were expressed in µg per g FW.

2.5. Data analyzes

Biochemical parameters (PROT content, LPO, PC, CAT, SOD, GPx, GSTs, ETs) and pigments were submitted to hypothesis testing using permutational multivariate analysis of variance, employing the software PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations. The pseudo-F values in the main tests were evaluated in terms of the significance and, when significant ($p < 0.05$), pairwise comparisons were performed.

For each descriptor, significant differences among areas were assessed. All descriptors were analyzed following a one-way hierarchical design, with sites or conserved and phase shift reefs as the main fixed factor. The null hypotheses tested were: a) for each biochemical parameter and for each pigment, no significant differences exist among reefs (R1 to R7); for each biochemical parameter and for each pigment, no significant differences exist among conserved (C) and phase shift reefs (PS). Significance levels ($p \leq 0.05$) among sampling reefs and stressed and not stressed reefs were presented with different letters.

Data from the biochemical parameters and pigments were transformed (square root), normalized and used to calculate an Euclidean matrix. This similarity matrix was simplified through the

241 calculation of the distance among centroids matrix based on reefs, which was then submitted to
242 ordination analysis, performed by Principal Coordinates (PCO).
243
244
245 **3. Results**
246
247 3.1. Cell damage
248 Organisms from three of the phase shift sites (R2, R3, and R4) showed the highest LPO levels and
249 thus with the most damaged membranes. At sites R5 and R6 (conserved) organisms have the
250 lowest LPO levels, although the difference is not significant from site R7 (conserved) and site R1 (in
251 phase shift) (Figure 2A). Comparing the overall LPO levels of organisms from phase shift sites with
252 non-impacted sites (Figure 2B), the formers had significantly higher values than the latter, showing
253 a higher degree of membrane stress in phase shift organisms.
254 Three of the phase shift sites (R1, R2 and R3) had lower PC levels than organisms from the other
255 sites, but the difference is not significant from the fourth phase shift site (R4). Organisms from
256 non-impacted (conserved) sites displayed identical PC levels ($p > 0.05$) (Figure 2C). The type of site
257 (in phase shift or conserved) influenced the level of protein carbonylation, with those from sites in
258 phase-shift displaying lower PC levels than conserved sites (Figure 2D).
259
260 3.2. Cell metabolism
261 ETS activity was identical in most of the sites studied, except for sites R1 and R4, which had
262 significantly higher ETS activities (Figure 3A). On average, organisms from phase shift sites had
263 higher ETS activity than those from non-impacted sites, showing the higher energy expenditure of
264 the first compared to the second (Figure 3B).
265 Soluble protein content is different between sites, being higher at R1, R2 and R3 and lower at R5
266 and R7 sites (Figure 3C). Although reef coral sites in different conservation state (R4 and R6) have
267 identical protein levels, the mean protein content of phase shift sites is significantly higher than in
268 conserved sites (Figure 3D).
269
270 3.3. Antioxidant and biotransformation response
271 Organisms from different sites showed significant differences in superoxide dismutase (SOD)
272 activity. In R1 and R3 organisms had high SOD activity, while identical activity ($p> 0.05$) was

observed among organisms from R5, R6 and R7 (conserved sites) that was smaller than activity in organisms from other sites in phase shift (R1 to R4) (Figure 4A). Thus, organisms from conserved sites showed significantly less SOD activity than from phase shift sites (Figure 4B).

The site of the organism's origin influenced CAT activity, with site R2 showing significantly lower activity than the other sites and sites R1, R3 and R5 higher CAT activity (Figure 4C). Since organisms from phase shift sites displayed the highest and lowest activity, there was no significant difference in CAT activity between organisms from phase shift and conserved sites (Figure 4D).

GPx activity showed no significant differences among organisms from different sites (Figures 4E and 4F).

Organisms from R1 and R4 showed higher GSTs activity than organisms from other sites, especially from site R2 (Figure 4G). Comparing the average response of organisms from conserved and phase shift sites, the former evidenced significantly less activity than the latter (Figure 4H).

3.4. Photosynthetic pigments

The host from conserved sites (R5, R6 and R7) and R1 showed higher chlorophyll a content than organisms in the remaining three sites (R2, R3 and R4), but significant differences were only found between R3 and R5 and R2 (Figure 5A). The chlorophyll a content of the hosts from conserved and phase shift sites showed that the former exhibited significantly more chlorophyll a than the latter (Figure 5B).

Chlorophyll c and peridinin did not show significant differences among organisms from different sites, and therefore, no differences between organisms from sites with different conservation status were either observed. However, three of the phase shift sites (R2, R3 and R4) had 3% to 88% lower chlorophyll c and 3% to 70% lower peridinin content than organisms from conserved sites, respectively. Organisms from the fourth phase shift site (R1) had similar (peridinin) or even higher (18 to 39%) chlorophyll c content than organisms from conserved sites.

3.5. Multivariate analysis

Principal Components Ordination (PCO) evidenced that together PCO1 and PCO2 explained 82.1% of the total variation obtained among *P.* cf. *variabilis* from seven different sites in BTS (Figure 6). Along PCO1, two groups were clearly separated, organisms from conserved sites (R5, R6 and R7) on the negative side of the axis and organisms from phase shift sites (R1, R2, R3 and R4) on the

positive side of the axis. PCO2, explained 32% of total variation, separating organisms from the four sites in phase shift, R2 in the positive side of axis 2, R3 and R4 next to the axis origin, and R1 on the far native side of axis 2. From PCO analysis it is possible to observe that PC and chlorophyll a were strongly correlated ($r > 0.85$) with organisms from conserved sites, while LPO, Prot and SOD were more correlated ($r > 0.85$) with organisms from phase shift sites, evidencing the metabolic shift of organisms at these sites, the induction of antioxidant response that was not able to restrain oxidative stress and damage overcame (LPO). Organisms from R1 site evidenced a different response, being highly correlated ($r > 0.85$) with ETS, CAT, SOD, GSTs and Chl c. Thus, evidencing a higher metabolic activity, antioxidant and biotransformation response and chlorophyll c content.

Discussion

Coral reefs phase shift, associated with loss of diversity and increase of non-reef builders such as soft corals, is worldwide documented (Burke et al., 2011, Cruz et al., 2015a, Done, 1992; Dutra, 2006, Edinger, 1998, Pandolfi et al., 2003, Riegl et al., 2009) and was related to environmental degradation caused by human activities and global changes (Downs et al., 2013; Hatcher et al., 1989, Nielsen et al., 2018; Pandolfi et al., 2003). However, little is known about the changes soft corals undergo and that trigger the population outbreaks.

An insight into the results obtained in our study confirmed differences in the biochemistry and metabolism of *Palythoa* cf. *variabilis* from conserved and phase shift sites, responding to the first aim proposed. Results also allowed to identify biochemical parameters recognizing corals in stress, which was the second aim of the study. Lastly, results also pointed out biochemical parameters reflecting the level of stress organisms are experiencing, answering to the third aim proposed for the study.

Our results evidenced a clear distinction in the biochemistry and metabolism of corals from conserved sites and sites in phase shift, and these changes may be the trigger for population outbreak. The organisms from the three conserved sites displayed similar biochemical features, with low oxidative stress, evidenced by the lower activity of antioxidant enzymes (especially SOD), lower damage (LPO), lower metabolic activity (ETS and protein) and a higher concentration of photosynthetic pigments, especially of chlorophyll a. Most sites in phase-shift simultaneously

exhibited a reduction in photosynthetic pigments and increased oxidative stress, not allowing to identify which of the two partners change first. Several studies (Downs et al., 2013; Krueger et al., 2014; Ladriere et al., 2008; Nielsen et al., 2018) reported that under stress conditions endosymbionts undergo photosynthetic changes before oxidative damage is settled. The deficient photosystem II activity was proposed as the primary source of oxidative stress, both in the dinoflagellate and the host (Downs et al., 2000; Downs et al., 2002; Lesser, 1996). On the contrary, Brown et al. (2002) evidenced the importance of the host's stress level (host antioxidant enzymes and heat shock proteins) in the symbiosis. Wooldridge (2013) also related the excess production of ROS beyond the antioxidant defence strategies of the coral host with host-cell necrosis and dinoflagellate expulsion. Nielsen et al. (2018) using a single-cell approach that maintained the coral-algae symbiosis observed that photosystems stress might in fact be a late-stage response in the bleaching process and not the initial driver of decreased number of endosymbionts. Results from our study showed that in one of the sites in phase-shift (R1) organisms presented oxidative stress without evidencing changes in photosynthetic pigment levels. Other studies (Buxton et al., 2012; Wooldridge, 2013) also suggested that the bleaching response is initially triggered by the dysfunction within the "dark reactions" of photosynthesis due to the failure of the coral host to maintain a sufficient supply of CO_2 for its endosymbiont partner (Buxton et al., 2012; Wooldridge, 2013). However, our results showed that ETS activity (which produces CO_2) is higher in corals from phase-shift than from conserved sites, thus refuting this suggestion. Considering these results, we can infer that in TSB the coral is the first to suffer the impact of environmental changes and to trigger antioxidant mechanisms and when is not able to control the stress it seems that the endosymbiont is then affected, however, further studies are necessary to confirm this assumption. Moreover, in the coral-dinoflagellate symbiosis the intracellular dinoflagellate microalgae supply photosynthetically fixed carbon to support the metabolism, growth, reproduction and survival of the coral host (Davies, 1991; Muscatine et al., 1984; Yellowlees et al., 2008). In return, dinoflagellates, among other benefits, have access to nutrients from the coral host, such as CO_2, inorganic nitrogen and phosphate (Davy et al., 2012). Despite the obvious benefits of this partnership to the host, the symbiosis with dinoflagellates may limit coral resources, since part of the nitrogenous compounds, needed in the synthesis of important macromolecules such as proteins, are shared between the endosymbiont and the host (Sutton and Hoegh-Guldberg, 1990; Trench 1971; Wang and Douglas 1999). If fewer nitrogen compounds are available, cellular processes in corals involving these compounds, such as protein synthesis, can be limited. Thus, the

synthesis of new proteins that will replace degraded (carbonylated) ones may be a less efficient process, leading to a higher level of protein carbonylation (Lehnigher et al., 2005). In fact, our results showed higher levels of protein carbonylation in organisms from non-impacted sites compared to phase shift ones. On the other hand, as organisms are not stressed, they do not need to activate metabolic pathways (enzymes) that adapt organisms to new conditions, such as increased oxidative stress (Matos et al., 2019; Pires et al., 2017), and therefore there is no need to increase protein levels.

Our results show that corals from phase shift sites presented biochemical and metabolic changes in line with those already reported, that studied the effect of various stresses on corals (Dias et al., 2019, Downs et al., 2013, Marques et al., 2020, Xiang et al. 2019), and therefore pointing to organisms from TSB in phase shift exhibiting differences in the biochemistry and metabolism compared to corals from conserved sites.

Our results also evidenced that some of the parameters determined can mark if corals are in stress. Corals from all phase shift sites presented similar values and activities in some of the biochemical parameters analyzed, such as higher protein content and higher SOD activity (Figure 6), thus these parameters can be used to discriminate between corals from conserved and phase shift sites. Since SOD is considered the first line of antioxidant defence (Fridovich, 1978) and the amount of soluble protein can be used to estimate the number of enzymes present in a cell (Lehnigher et al., 2005), both parameters evidence the cell's effort to induce mechanisms adapting cells to the new prevailing conditions, such as combating oxidative stress with the induction of antioxidant enzymes (SOD activity). Several studies reported biochemical changes in corals exposed to constraints such as contamination (Marques et al. 2020, Xiang et al. 2019), temperature rise (Dias et al. 2019, Downs et al. 2002, Downs et al. 2013), variation in light intensity (Downs et al. 2013) and salinity changes (Dias et al. 2019). Exposure to contaminants such as benzo[a]pyrene (Xiang et al. 2019) decreased chlorophyll a, causing oxidative stress, which was combated by increasing the activity of antioxidant enzymes such as SOD, and changed the levels of HSP70 (protein chaperons related to protein conformation and stability). Exposure to Cu alone or in combination with other stresses (ocean acidification and temperature rise) led to changes in chlorophyll a (Marques et al. 2020) and inhibited the activity of enzymes linked to the Krebs cycle and fermentation and the electron transport chain (Fonseca et al. 2019). The increase in temperature and light intensity altered chlorophylls and carotenoids, increased protein

carbonylation and toxic aldehydes (originated in lipid peroxidation), increased ubiquitin (linked to protein degradation) and the activity of antioxidant enzymes such as glutathione reductase and SOD (Downs et al. 2013). When assessing the influence of increased temperature and decreased salinity, Dias et al. (2019) found that temperature increase caused damage to the membranes (increased LPO) and induced the response of antioxidant enzymes with increased activity of SOD, CAT and also the biotransformation response (GSTs), whereas lower salinity had the opposite effect. Our results show that corals from phase shift sites all have higher antioxidant enzyme (SOD) activity and this parameter, as well as protein content and protein carbonylation, can be used to assess disturbance in coral reefs.

Our results also allowed to identify parameters that can reflect the level of stress corals are experiencing. Differences in some biochemical endpoints among organisms in phase shift but from different sites were also observed, indicating that the degree of stress can be different among corals in phase shift. Variations in GSTs activity, among corals in phase shift, were noticeable, with corals from R1 displaying the highest and corals from R2 site the lowest GSTs activity. Aldehydes can be formed from peroxidized polyunsaturated fatty acids by the action of hydroperoxidelyases (Cardoso et al., 2017; El-Aal, 2012). The GSTs conjugation activity of aldehydes with glutathione originates less reactive compounds, reducing their interaction with proteins and nucleic acids (Lemire et al., 2013) and contributing to increasing the tolerance to oxidative stress. Thus, the increase in GSTs activity in organisms from R1 site may explain the similar LPO levels to organisms from conserved sites and lower than those observed in other phase shift sites. Thus, corals from this site (R1) seem to cope with oxidative stress, as they present low LPO levels and were able to maintain chlorophyll levels identical to organisms from conserved sites. On the other hand, corals from R2 site presented low GSTs and CAT activity, even lower than corals from conserved sites, but the damage (LPO and PC) is similar to organisms from other sites in phase shift. The lowest levels of photosynthetic pigments especially chlorophyll a (45%) and lower ETS activity (about 15%), the main processes of ROS generation in the cell (Foyer and Noctor, 2003), observed in organisms from R2 site, may have contributed to decrease the overall ROS concentration in cells, compensating the generation of ROS by exogenous causes and preventing cell damage (LPO) from being higher than in organisms from other phase shift sites. Results from the present study evidenced GSTs and CAT activity as endpoints differing among corals from different sites in phase shift, and that can be used to recognize the level of stress organisms in phase shift are experiencing.

Conclusion

Overall, the results obtained in the present study evidenced that the ability of corals to restrain oxidative stress in the host cell and to protect the endosymbiont seems to be an important feature in the maintenance of symbiosis with dinoflagellates. Results also allowed to relate oxidative stress in *P. cf. variabilis* with phase shift and to identify parameters that can discriminate between stressed and non-stressed reefs. Moreover, organisms from disturbed sites (in phase shift) were not all at the same level of oxidative stress, and biochemical endpoints were able to mark differences in the stress level organisms are experiencing.

Thus, the biochemical patterns of *P. cf. variabilis* can be used to predict coral reef conservation state in TSB. Some of the parameters were able to discriminate more subtle and more noticeable changes and may allow to recognize the most at-risk coral reefs that need immediate intervention and prevent the entry into or revert *P. cf. variabilis* outbreak and phase shift in coral reefs. Actions like these can be of vital importance for the preservation of TSB coral reefs and possibly for other threatened reefs worldwide.

Acknowledgments

The authors would like to thank Jorge Galvão for his invaluable help during the sampling campaign. This work was financially supported by the grant EC-187C-18 - Phase Shift on Todos os Santos Bay / Brazil: restoring ocean health for biodiversity funded by National Geographic Society (NGS). Thanks are due to FCT/MCTES for the financial support to CESAM (UID/AMB/50017/2019), through national funds. AP was contracted by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. This work was also financially supported by the project BIOGEOCLIM (POCI-01-0145-FEDER-029185) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES.

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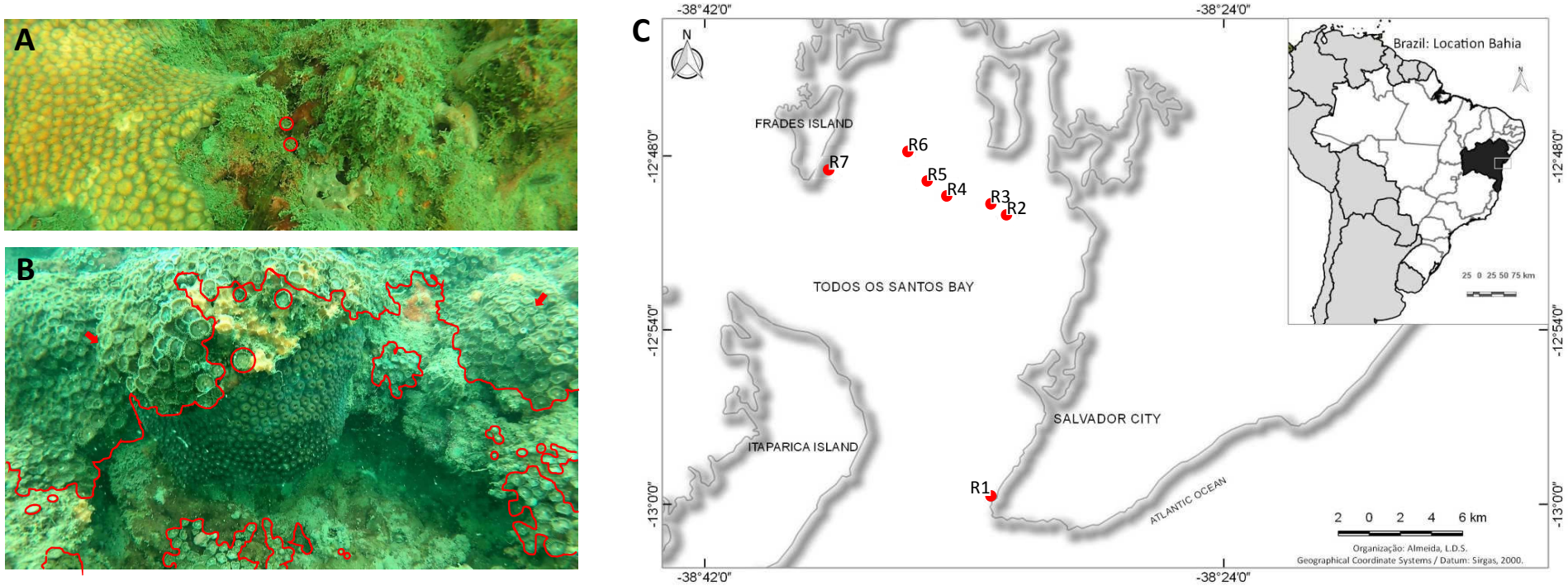

Fig. 1 – Coral reefs and study area. Examples of a conserved coral reef (A) and of a coral reef at phase shift (B). Areas covered by *Palythoa* cf*. variabilis* are outlined by a red line. Location of the sampling sites (R1 to R7) (A) in Todos os Santos Bay (TSB). Fig. 1C adapted from Campos and Figueira, 2019

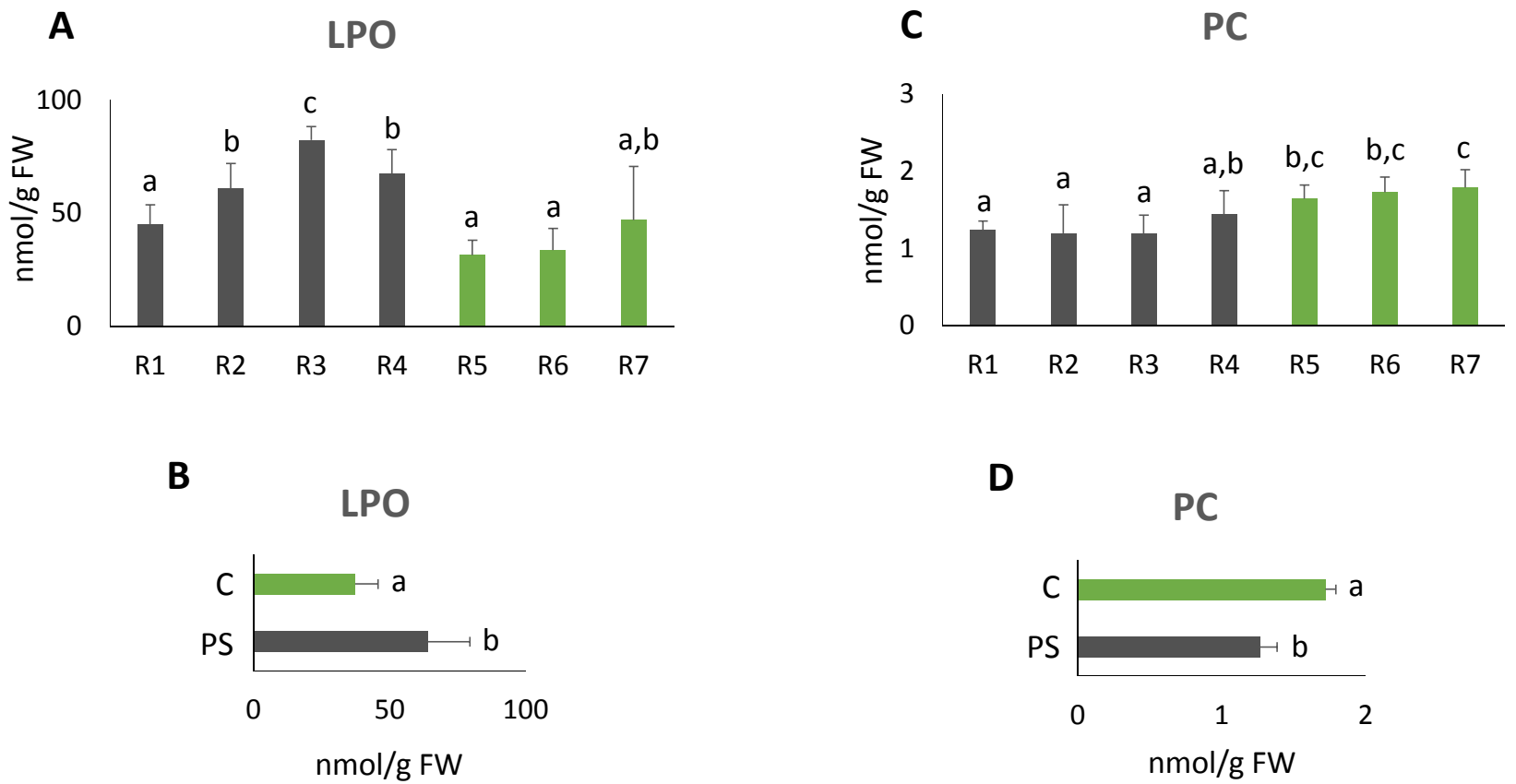

Fig. 2 - Cell damage. Lipid peroxidation, LPO (A and B), and protein carbonylation, PC (C and D) in *Palythoa* cf. *variabilis* individuals collected from seven sampling sites in TSB (A and C) and from conserved reefs (C) and reefs in phase shift (PS) (B and D). Significant differences ($p \leq 0.05$) among areas and among C and PS reefs are presented with different letters (a–d). Reefs in phase shift (R1, R2, R3 and R4) are represented by gray bars, conserved reefs (R5, R6 and R7) are represented by green bars.

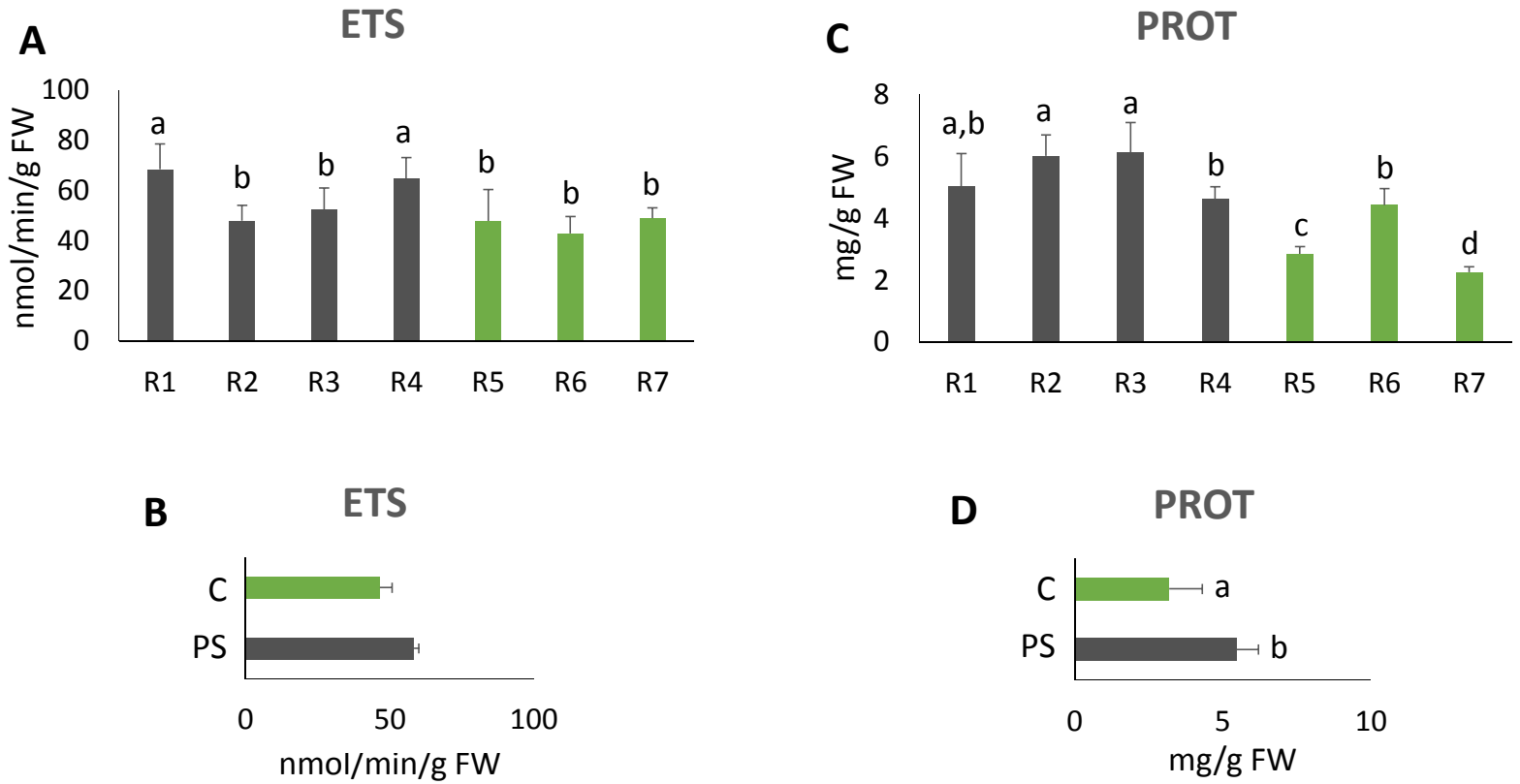

Fig. 3 - Electron transport chain (ETS) (A and B) and total protein (PROT) (C and D) mean values (±standard deviation), in *Palythoa* cf. *variabilis* individuals collected from seven sampling sites in TSB (A and C) and from conserved reefs (C) and reefs in phase shift (PS) (B and D). Significant differences ($p \leq 0.05$) among areas and among C and PS reefs are presented with different letters (a–d). Reefs in phase shift (R1, R2, R3 and R4) are represented by gray bars, conserved reefs (R5, R6 and R7) are represented by green bars.

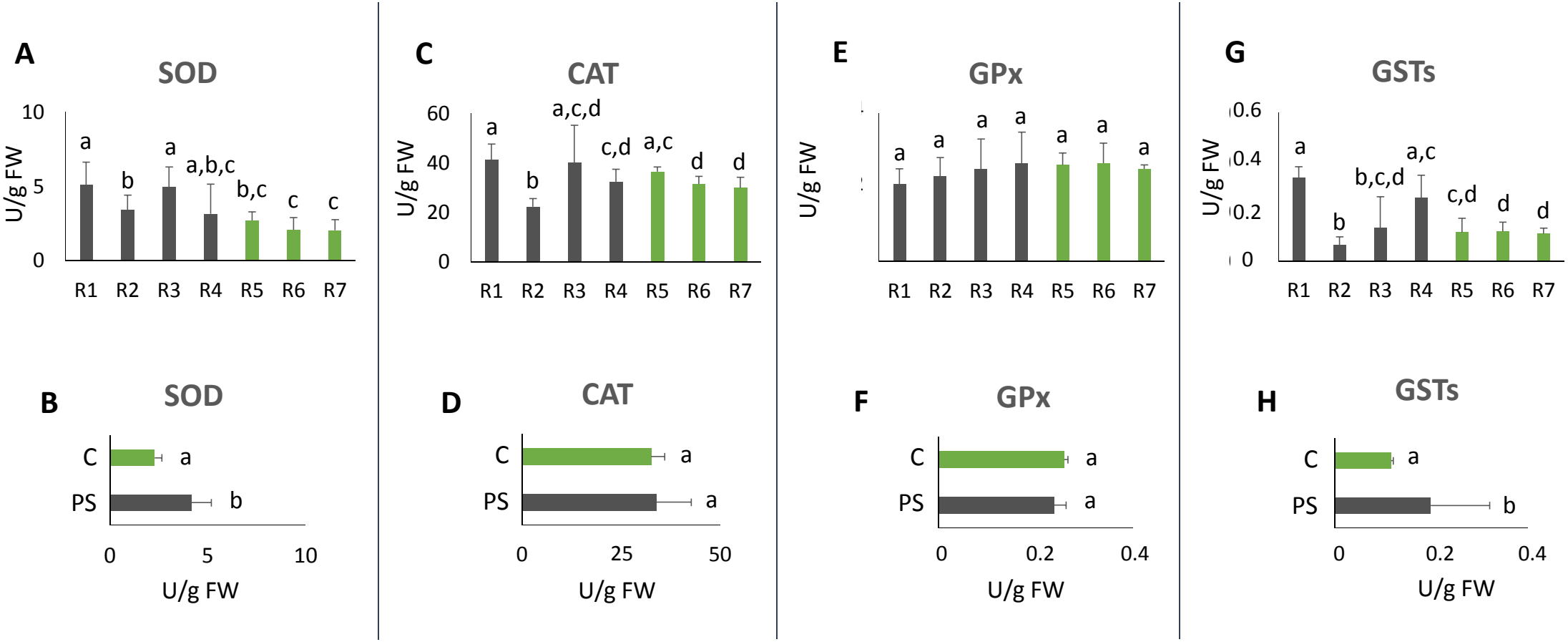

Fig. 4 - Antioxidant and biotransformation enzymes. Superoxide dismutase, SOD (A and B); Catalase, CAT (C and D); glutathione peroxidase, GPx (E and F);); glutathione-S- transferases, GSTs (G and H) in *Palythoa* cf. *variabilis* individuals collected from seven sampling sites in TSB (A, C, E and G) and from conserved reefs (C) and reefs in phase shift (PS) (B, D, F and H). Significant differences ($p \leq 0.05$) among areas and among C and PS reefs are presented with different letters (a–d). Reefs in phase shift (R1, R2, R3 and R4) are represented by gray bars, conserved reefs (R5, R6 and R7) are represented by green bars.

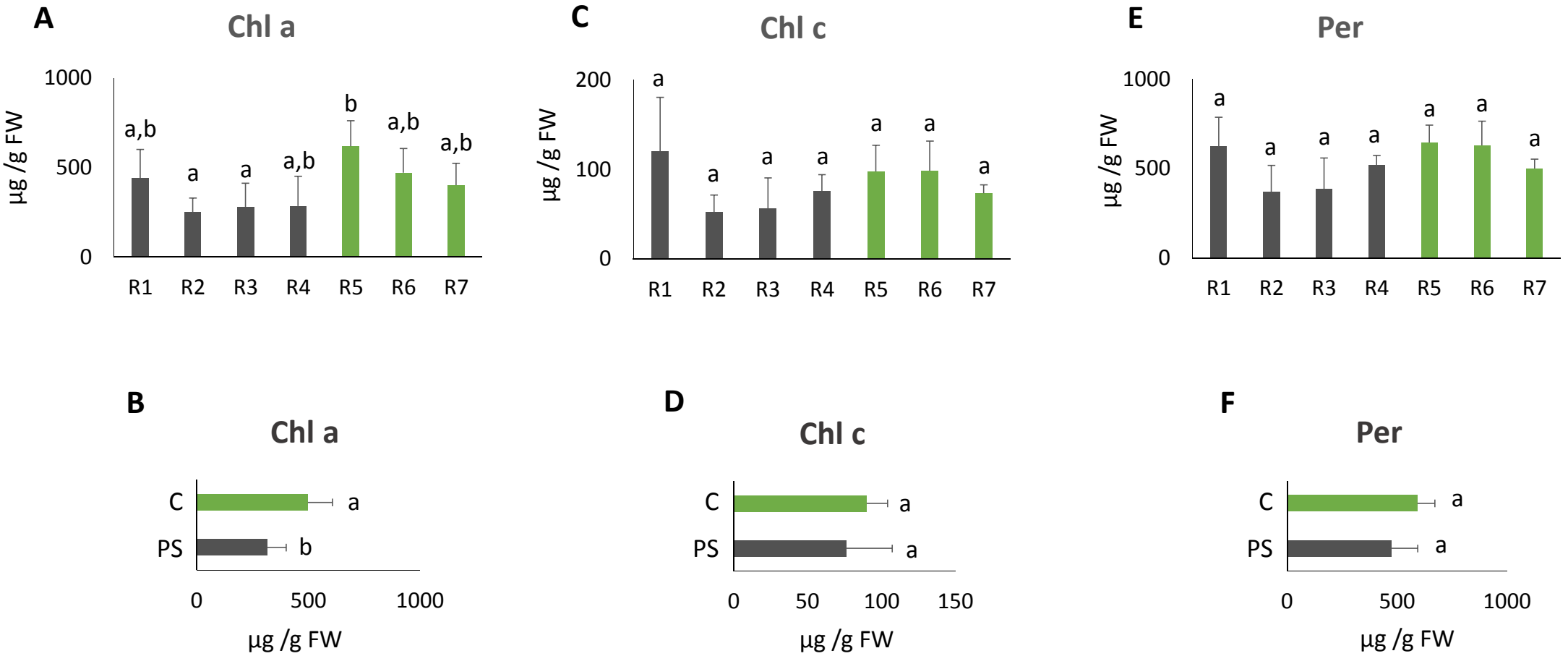

Fig. 5 - Photosynthetic pigments from symbiotic dinoflagellates. Chlorophyll a, Chl a (A and B), chlorophylls c, Chl c (C and D) and peridinin, Per (E and F) mean values (±standard deviation) in *Palythoa* cf. *variabilis* individuals collected from seven sampling sites in TSB (A, C and E) and and from conserved reefs (C) and reefs in phase shift (PS) (B, D and F). Significant differences ($p \leq 0.05$) among areas are presented with different letters (a–d). Reefs in phase shift (R1, R2, R3 and R4) are represented by gray bars, conserved reefs (R5, R6 and R7) are represented by green bars.

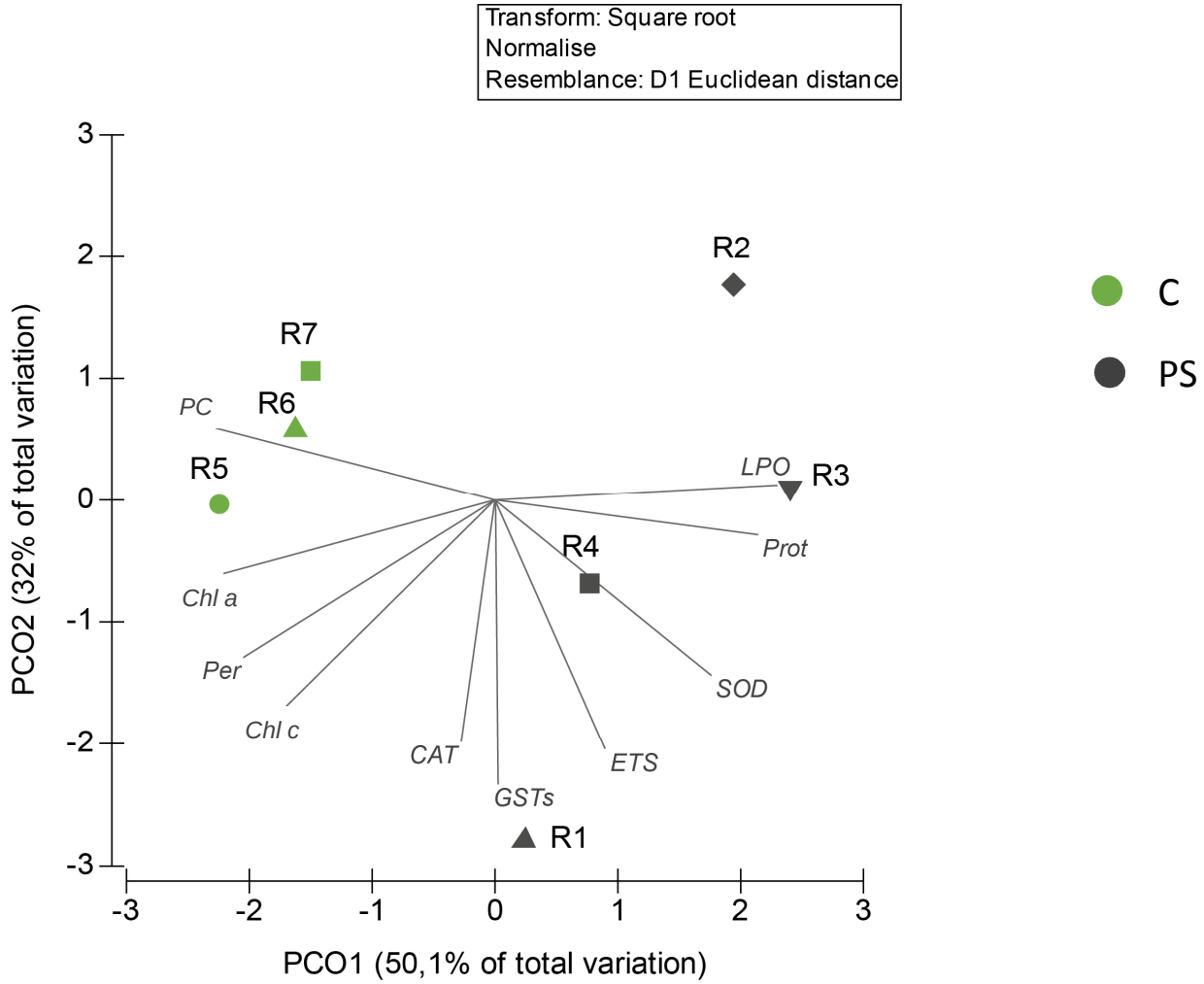

Fig. 6 - Centroids ordination diagram (PCO) based on biochemical parameters and pigment concentrations in *Palythoa* cf. *variabilis* collected from seven sampling sites in TSB. Pearson correlation vectors are superimposed as supplementary variables ($r > 0.85$): (CAT) catalase activity; (Chl a) chlorophyll a concentration; (Chl c) chlorophyll c concentration; (GSTs) glutathione S-transferases activity; (LPO) lipid peroxidation concentration; (Per) peridinin concentration; (PC) Protein carbonylation concentration; (SOD) superoxide dismutase activity. Reefs in phase shift (R1 to R4) are represented in gray and conserved reefs (R5 to R7) are represented in green colors.

Highlights

- *P.* cf. *variabilis* from reefs in different conserved states display distinct biochemical patterns
- Higher Protein carbonylation appears as a hallmark of *P.* cf. *variabilis* from conserved reefs
- Higher total protein, lipid peroxidation and Superoxide dismutase activity can identify reefs in phase shift
- Glutathione S-Transferases activity can be a predictor of different stress levels in phase shift reefs

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: