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EFFECT OF SEA SURFACE TEMPERATURE ON CATALASE AND GLUTATHIONE S-TRANSFERASE ACTIVITIES IN SCLERACTINIAN CORAL *Acropora digitifera* FROM PULAU BIDONG, TERENGGANU

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Abstract: Natural exposure of coral reefs to stressors induces an oxidative stress response. In Malaysia, biomarkers used to assess this response are limited. This study investigated the activities of the antioxidant enzymes glutathione S-transferase (GST) and catalase (CAT) in the scleractinian coral *Acropora digitifera* across three separate sites in Pulau Bidong, Terengganu during March, May, July, and September 2018 to understand the variations in these enzyme activities. Fragments of corals were airbrushed to remove tissue, disrupted to release cytosol, and the enzyme activities were assayed. The result demonstrated variation in GST activities with the highest level in May ($p < 0.05$) and in contrast, there was no significant difference in CAT activities between different sampling months and sites ($p > 0.05$). In addition, *A. digitifera* collected in Pantai Pasir Cina (PPC) showed the highest antioxidant activities for both enzymes (GST, 19.6 ± 5.10 µmol/min/mg protein; CAT, 14.6 ± 3.40 U/mg protein), suggesting corals in this location are responding to oxidative stress. These values are lower than previously reported for *A. digitifera* in Pulau Bidong, indicating a stable and adequate baseline level of antioxidant enzyme activities. These findings provide valuable insights and crucial information for future reference.

Keywords: Antioxidant enzymes, biomarkers, oxidative stress, free radicals, reactive oxygen species.

Introduction

The coral reef area undeniably holds a huge capability to contribute significantly as the life support system of the earth. Coral reefs supply many goods and services such as seafood, shoreline protection, carbon dioxide fixation, and recreational activities (Crossland *et al*., 1991; Moberg & Folke, 1999). In terms of the fisheries industry, Southeast Asia dominates over a quarter of the world's small-scale fishers on coral reefs and half of the coral reef fish is in this region, thus, supporting the socioeconomic well-being of the coastal communities (Teh *et al*., 2011; 2013). In Malaysia, coral reefs cover an area of 4,006 km^2 with over 550 species contributing to the economy of the country (Praveena *et al*., 2012). Therefore, Malaysia is listed as one of the countries in the Coral Triangle, which is defined as an area that is known to be rich in marine biodiversity and home to 76% of all known coral species and 53% of the world's coral reefs (Cros *et al*., 2014; Khodzori *et al*., 2019). Through a management tool such as the establishment of marine parks in 1983 under Marine Protected Area (MPA), the Department of Fisheries Malaysia (DOF) is trying to protect, conserve, and manage the coral reefs area and its biological diversity to ensure sustainable advantages from fisheries and tourism (Gazi *et al*., 2013; Masud *et al*., 2017). According to the Department of Marine Park Malaysia, based on the Total Economic Value (TEV) of Malaysia Marine Park from 2011 to 2015, the establishment of marine parks gave benefits that covered a wide range of ecosystem services and have been estimated to achieve a value of RM8.7 billion (Jabatan Taman Laut Malaysia, n.d.).

Despite the benefits and value, the coral reef ecosystem is threatened and their abundance is declining rapidly worldwide as a result of regional natural impacts (e.g., overexploitation of fisheries, declining water quality, coral bleaching, and tropical storms), global natural impacts (e.g., global warming and ocean acidification), and most importantly human impacts (e.g., tourism and anthropogenic activities) (Wilkinson, 2000; Hoegh-Guldberg *et al*., 2007). Unprecedented natural events such as global warming and storms can worsen conditions in specific areas (Guest *et al.,* 2012; Safuan *et al.*, 2020). A major concern is that the rapid changes in the environment could surpass the evolutionary ability of the coral to adapt and the collapse of coral reef ecosystems could have consequences, especially for the reef-based fisheries industry and biodiversity (Cesar *et al*., 2003; Graham *et al*., 2013; Pratchett *et al*., 2014). Undoubtedly, the coral reef ecosystem will be different in the future than it is currently; thus, actions must be taken to tackle the issue and ensure this ecosystem persists in the long term.

The research on oxidative stress in coral reef biology started to emerge in the hope that it could lead to a better understanding of this critical situation. Oxidative stress has been defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defence, which results in cellular damage (Sies, 1997; Betteridge, 2000). A wide range of antioxidant enzymes such as catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD) are synthesised by aerobic organisms in an attempt to reduce oxidative stress damage as it could affect biologically important molecules such as protein, carbohydrates, lipids, and nucleic acids (DNA and RNA) (Davies, 1995; Rabilloud *et al.*, 2005). Scleractinian coral, which plays a vital role in reef habitat structuring can also not escape from the fall-off in populations as a response to various stressors (Wild *et al*., 2011). Scleractinian corals and other corals are greatly dependent on the symbiotic algae called zooxanthellae which under stress, their symbionts are expelled from the host coral, a process known as coral bleaching (Hoegh-Guldberg *et al*., 2007; Baird *et al*., 2009; Shafiqa-Yusof & Radzi, 2022).

In investigating the impact of oxidative stress on coral, a biomarker technique has been employed to measure the diminished biological responses, including physiological and biochemical changes in corals (Lesser, 2006). Generally, biomarkers have been widely used for decades as a tool in environmental monitoring, especially in marine environments. More studies are focusing on biomarkers of contaminants or pollutions in the aquatic habitat whereby contaminants or pollutions were exposed to the organisms in the laboratory setup (Monserrat *et al*., 2011; Schettino *et al*., 2012; Valon *et al*., 2013). Likewise, biomarker responses can be measured in organisms that are collected from or deployed on the field sites and afterwards, the effects of chemical and non-chemical stressors are examined (Hook *et al*., 2014).

This study focused on determining the cellular stress response of the coral *A. digitifera* using antioxidant enzymes as biomarkers. This study assessed the oxidative stress response in this commonly found scleractinian coral around Pulau Bidong, Terengganu by evaluating the activities of two antioxidant enzymes, glutathione S-transferase (GST) and catalase (CAT). Enzyme GST is important in stress response as it is involved in detoxification (Downs *et al*., 2005). Meanwhile, CAT is the most effective enzyme in scavenging hydrogen peroxide (H_2O_2) and converting it to non-harmful products (Lesser, 2006). We hypothesised that the activities of enzymes CAT and GST in the wild population of *A. digitifera* in Pulau Bidong, Terengganu will exhibit significant variations, reflecting the oxidative stress response to environmental stressors. By providing both enzyme activities, the research contributes valuable insights into oxidative stress responses on coral health and serves as a crucial data reference for coral reef conservation and restoration efforts in Malaysia.

Materials and Methods

Site Description and Collection of Samples

Acropora digitifera were collected from three sites within the reefs of Pulau Bidong, Terengganu, Malaysia (Figure 1). The Pulau Bidong reef is covered by 51.98% live coral based on six sites surveyed. From 1978 to 1991, Pulau Bidong was a centre for Vietnamese refugees and has remained unpopulated since. Field sampling was conducted at three sites in Pulau Bidong in March, May, July, and September 2018, the selection of sites was based on the availability of distant coral colonies at similar depths and non-shaded areas. The first site was at Pantai Pasir Cina (PPC, 5.62°N, 103.05°E), in which there is a research station of Universiti Malaysia Terengganu (UMT). The second site was at the Pantai Pasir Pengkalan (PPP, 5.36°N, 103.35°E), where the jetty is located and frequently visited for educational and tourism purposes. The third site was at the Pantai Tenggara (PT, 5.61°N, 103.06°E), where the underwater coral garden of Yayasan Coral Malaysia receives minimal human activities. At each site, samples of *A. digitifera* were tagged and collected, consisting of three fragments as biological replicates with length ~5 cm, taken from three separate colonies at depths around five to seven metres. The sea surface temperature (SST) was recorded throughout the year from January to September using the HOBO U20l-01 research-grade water level data logger (Onset Computer Corporation, Bourne, MA). Data was missing from October to December because of logger issues. The samples collected were kept in sealed bags underwater. Water was removed from the bags on the deck and samples were immediately transferred to an icebox. In the laboratory, samples were stored in a -80°C freezer until analysis.

Coral Tissue Extraction

Coral tissue containing zooxanthellae was removed from the skeleton by using an airbrush containing distilled water within a small sealed

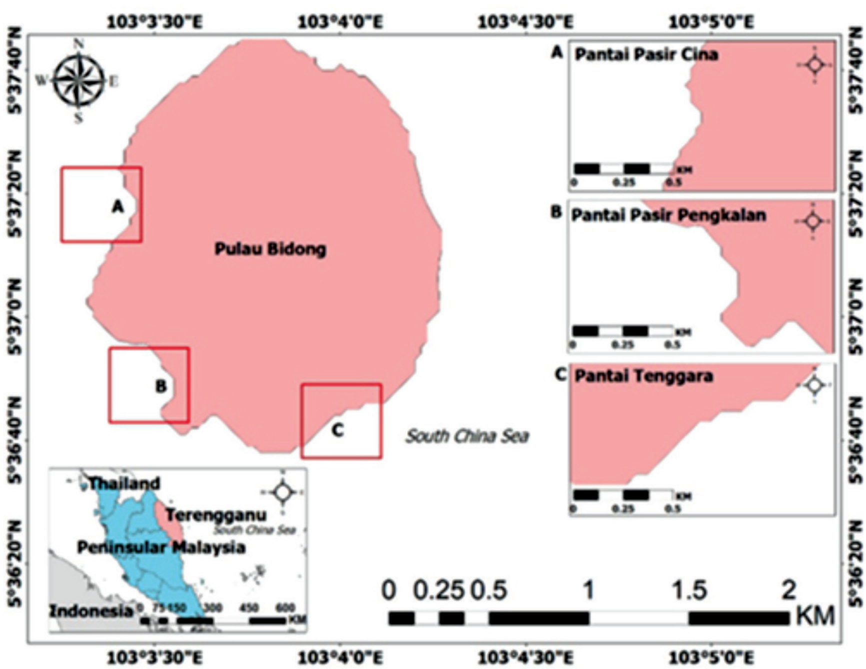

Figure 1: Location of the study sites at Pulau Bidong in Terengganu, Malaysia. (A) Pantai Pasir Cina, PPC, (B) Pantai Pasir Pengkalan, PPP, and (C) Pantai Tenggara, PT

plastic bag to prevent loss by splattering (Figure 2). The resulting tissue suspended in water was poured into a 15 mL centrifuge tube and samples were centrifuged (7,000 × g for 10 minutes at 4°C). The resulting supernatant was removed and the zooxanthellae pellet was stored in a -80°C freezer until further analysis was performed. All subsequent assays were run in triplicate (technical replicates).

Protein Concentration Determination

Before conducting total protein determination and enzymatic analysis, the stored pellets were suspended in homogenise buffer containing 10 mM Tris HCl, 500 mM sucrose, 1 mM EDTA, and 0.15 M KCl, pH 7.2 and sonicated on ice (three times, 10 minutes each). After sonication, the sample was homogenised with an ultrasonic cell disruptor (Misonix, Inc., Farmingdale, New York) for 20 seconds and 10 cycles each. The sample was once again centrifuged at 6,000 × g, 4°C for 15 minutes and supernatant was placed in new Eppendorf tubes. The protein concentration was estimated by the Bradford method (Bradford, 1976) to normalise the enzyme activity results. The total protein concentration of the coral tissue was expressed in mg/mL.

Antioxidant Enzyme Analysis

CAT activity was measured using the method described by Beers and Sizer (1952) in which the decreases in H_2O_2 concentration were evaluated spectrophotometrically at 240 nm. The working solution of 0.05 M phosphate buffer with pH 7, 0.059 M H_2O_2 (30%), and sample extract was mixed in a cuvette. The UV-1800 UV-Vis Spectrophotometer (Shimadzu, Tokyo, Japan) was adjusted to 240 nm, 25°C, and the decrease in absorbance was recorded for 120 seconds.

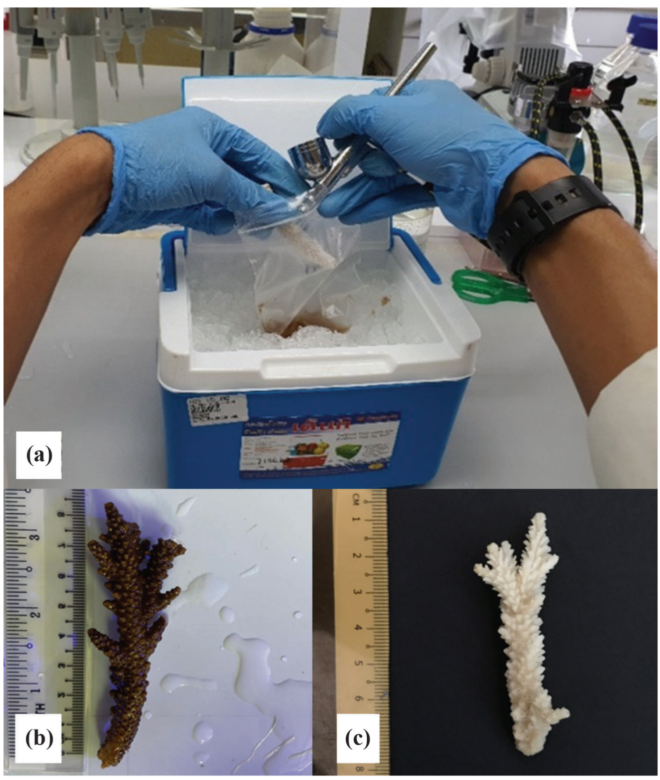

Figure 2: Airbrush technique was used for coral tissue extraction from the skeleton. (a) Tissue was removed within the plastic bag and on ice to prevent protein degradation, (b) coral skeleton with intact tissue, and (c) Coral skeleton condition after the tissue removal

The activity was expressed as U/mg protein. One unit (U) of CAT activity is defined as the amount of enzyme needed to decompose one micromole of H_2O_2 per minute (1 µmol $H_2O_2 \cdot min^{-1}$). The following formula was used to calculate the CAT enzyme activity:

$$\text{CAT activity (U/mg protein)} = \frac{\Delta A240\ min^{-1} \times 1000}{43.6\ M^{-1} cm^{-1} \times \text{mg enzyme}} \quad (1)$$

where:

mg enzyme = weight of enzyme added in 1.5 mL total reaction volume
$43.6\ M^{-1}\ cm^{-1}$ = extinction coefficient for H_2O_2 at A240 nm
1.5 mL = volume of reaction
$\Delta A240\ min^{-1}$ = change of absorbance at 240 nm per minute

Meanwhile, GST activity was determined by measuring the rate of conjugate produced between 1-cloro-2,4-dinitrobenzine (CDNB) as a substrate and reduced glutathione (Habig *et al*., 1974; Jaafar *et al*., 2015). The working solution of 0.15 M potassium phosphate buffer pH 6.5, 2 mM 1-cloro-2,4-dinitrobenzine (CDNB), sample extract, and 20 mM reduced glutathione (GSH) was mixed in a 96-well plate. The activity was observed at 340 nm and the change in absorbance per minute was calculated for a total of five minutes ($\Delta A340$, $\varepsilon = 9.6 \cdot mM^{-1} \cdot cm^{-1}$). The changes in absorbance value were measured using the multimode microplate reader SpectraMax® iD3 (Molecular Devices, San Jose, California). The GST activity is defined as the amount of enzyme needed to synthesise 1 µmol of CDNB per minute and expressed as µmol/min/mg protein. The following formula was used to calculate the GST enzyme activity:

$$\text{GST activity (µmol/min/mg protein)} = \frac{\Delta A340\ min^{-1} \times 0.2\ mL \times df}{\varepsilon \times 0.524\ cm \times A} \times \text{prot. conc.} \quad (2)$$

where:

df = dilution factor of sample in the total volume of reaction
ε = extinction coefficient for CDNB conjugate at A340 nm
A = volume of samples in mL
$\Delta A340$ = change of absorbance at 340 nm per minute
0.2 mL = total volume of reaction
0.524 cm = distance for light travel in total volume of reaction

Statistical Analysis

The effect of factors (sites and months) was analysed using a two-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) test for multiple comparisons post hoc. All data were tested for homogeneity of variances using Levene's test before the ANOVA was carried out. Results were presented as means ± SD and the difference was considered statistically significant at $p < 0.05$. All statistical analysis was performed using SPSS v20.0 (SPSS Inc., Chicago, Illinois).

Results

Sea Surface Temperature (SST)

There was no visible bleaching observed throughout the sampling. The SST was recorded throughout the year from January to September 2018 by using a temperature logger. The logger was deployed on PPP which was one of the sampling sites in this study, assuming it could also represent the SST at the other two sampling sites which were located nearby (< 1 km) from this deploy area. The development of SST is discussed based on monthly averages. The SST at the location started in January with a mean temperature of 27.90 ± 0.22°C and gradually increased after that to the highest at 30.60 ± 0.19°C in May (Figure 3). Then, SSTs in this area maintained above 30°C with little fluctuation (0.02°C to 0.09°C) throughout the months. For instance, in June, the average

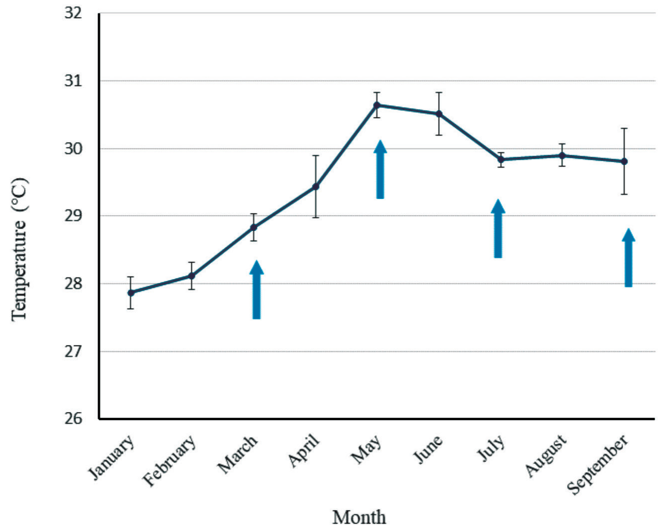

Figure 3: Plots of monthly averages of sea surface temperature, SST (± SE) from January to September 2018 recorded in Pulau Bidong. Missing data from October to December was due to the logger issues. The arrows indicate the sampling periods

SST was 30.50 ± 0.31°C and only dropped by 0.01°C from the previous month. After June, the SST slightly decreased and remained at 29.8°C to 29.9°C over the following months.

Antioxidant Enzymes Activities

In general, a two-way ANOVA test showed a statistically significant influence of both sampling months and sites on GST activities ($p < 0.05$). As presented in Figure 4 (a), coral tissue collected at PPC in May showed the highest GST activity (19.6 ± 5.10 µmol/min/mg protein) and significantly different ($p < 0.05$), with July showing the lowest GST activity (3.3 ± 0.96 µmol/min/mg protein). PPC also displayed a significant difference in GST activity compared to other sampling sites in May and September ($p < 0.05$).

On the contrary, based on the two-way ANOVA analysis, there was no statistically significant differences ($p > 0.05$) were observed in CAT activity for the interaction of both sampling months and sites. CAT activity displayed a stable level of activities across months of sampling in each sampling site compared to GST activities that expressed different patterns [Figure 4 (b)]. The highest CAT activity was in May at 14.6 ± 3.40 U/mg protein while the lowest was in July at 7.5 ± 1.57 U/mg protein. The response of CAT activity at PPC was the lowest and significantly different among other months and sites [$p < 0.05$, Figure 4 (b)].

Discussion

Reactive oxygen species (ROS) production in coral occurs in the chloroplast of the zooxanthellae by several mechanisms involving electron transfer catalysed by photosystems I and II, and the ROS further leads to the photoinhibition of photosynthesis in the zooxanthellae (Weis, 2008). The enzymatic antioxidant systems, which include GST and CAT play a crucial role in protecting biological systems against oxidative damage by ROS. Thus, assaying antioxidant systems might serve as oxidative stress biomarkers to monitor coral health (Downs *et al*., 2000). CAT is an important antioxidant enzyme that is a first-line defence against H_2O_2, limiting ROS-mediated damage to organisms. However, ROS action can be overwhelming; therefore, second-line defence against ROS is provided by enzymes such as GST (Lushchak, 2011; Regoli & Giuliani, 2014).

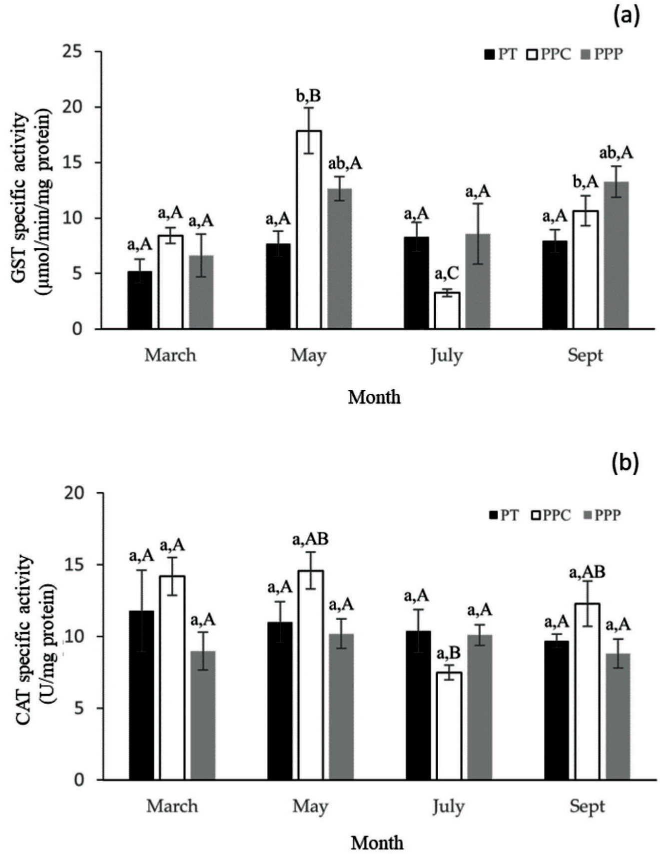

Figure 4: (a) GST (expressed in µmol/min/mg protein) and (b) CAT (expressed in U/mg protein) activities in *A. digitifera* collected at four different months of sampling from three different sites in Pulau Bidong. Data are expressed as mean ± SD. Superscripts of different lowercase letters indicate significant differences at the same months and different sites ($p < 0.05$) and different capital letters indicate significant differences at the same site and different months ($p < 0.05$)

In this study, CAT activities showed no significant differences throughout different months of sampling and between different sites ($p > 0.05$). Based on the response of the enzyme activities, we suggest that the coral *A. digitifera* eliminates H_2O_2 efficiently by the antioxidant enzyme CAT, as it is the major common intermediate and oxidant for a lot of biological molecules (Davies, 1995; Munoz-Munoz *et al*., 2009), therefore resulting in no significant variation in the activities. As mentioned earlier, CAT is a highly efficient and first-line defence antioxidant enzyme abundant in the peroxisomes of almost all living tissues, including corals and their symbionts, zooxanthellae. It breaks H_2O_2 into water and molecular oxygen, preventing cellular damage (Krueger *et al*., 2014; Ighodaro & Akinloye, 2018). Moreover, according to Winterbourne (2008), photosynthetically-derived H_2O_2 has been suggested as the most likely ROS to travel through cell membranes between both coral and zooxanthellae, as it has a longer lifetime than other ROS, a large diffusion radius, and no net charge. Another study by Lopes *et al*. (2018) reported that the coral species *Veretillum cynomorium* showed no significant difference in CAT activities. Due to the daily exposure to extreme changes in abiotic parameters such as temperature and location at the shallow reef, the coral species may have a higher tolerance to stressors (Lopes *et al*., 2018). Corals inhabiting shallow reef areas experience daily temperature fluctuations, which may account for the lack of significant variation across sites and months in this study. Additionally, a study by Samshuri *et al.* (2023) found that CAT and GST levels in *A. digitifera* from Pulau Bidong were higher and more varied across sampling sessions compared to this study. This condition was expected as the temperatures recorded in this study (27.9°C to 30.6°C) were lower than those in Samshuri *et al.* (2023), which were between 28.17°C and 33.73°C. This could indicate a steady and sufficient baseline level of antioxidant enzyme activities, particularly CAT, expressed by the corals. This has also been suggested in previous studies using antioxidant enzymes. In their research, the peroxidase activity showed no variation among experimental treatments for any coral species, signifying that the baseline levels of this antioxidant were adequate to prevent oxidative stress in the coral, in other words, the threshold of the stressors was not induced (Mydlarz & Harvell, 2007; Palmer *et al*., 2011).

Furthermore, this study displayed the highest activity for CAT and GST in coral samples collected at PPC. The condition of the site itself could explain this elevation. PPC is currently a research station for UMT. Many research activities involving fish and coral diversity such as snorkelling and diving (recreational activities) were conducted here (Daud & Akhir, 2015). There was also a possibility of underground sewage discharge from the station into the seawater on the site. Hence, human activities in this area may cause stressors to the coral reef ecosystems and these were supported by some of the previous studies that highlighted the same issue (Sandin *et al*., 2008; Kittinger *et al*., 2012). These stressors could lead to high antioxidant activities in coral to defend against oxidative stress. Besides, GST is known as a phase II biotransformation enzyme that catalyses the conjugation of xenobiotic compounds and has been used as a biomarker of exposure to environmental pollution in the organism (Fitzpatrick *et al*., 1997; Jaafar *et al*., 2015). The sewage discharge from the research station could be the source of anthropogenic pollutants that affect the coral reef on the PPC through the runoff or channels nearby. Considering the distance of the possible source of sewage that is close to the coral reef, it can cause harm as it has the potential to travel underground (Häder *et al*., 2020). The sewage discharge may contain personal care products, waste from kitchen sinks, and freshwater runoff from the drains that could affect the corals in the area.

The present study also showed the highest and most significant difference in GST activity ($p < 0.05$) in *A. digitifera* from May sampling compared to other months of sampling. It can be suggested that the elevation of GST activity, alongside CAT activity could indicate a defence of coral against oxidative stress damage by environmental stressors. The highest GST activity in May is probably linked to the highest SST recorded during the sampling month (Figure 3). This study is located in the South China Sea (SCS), which is influenced by the monsoon season, which affects the SST. SST is lowest in January and February due to the northeast monsoon affecting the area, bringing significant wind, waves, and rainfall. It then rises dramatically, reaching its peak in May as sunny weather begins, indicating the southwest monsoon (Daud & Akhir, 2015). The SST data in this study is consistent with a previous study by Daud *et al*. (2019) on the same region, whereby higher SST was spotted in May and June while lower SST appeared in January and February. May is normally the hot and dry weather before the monsoon. The same study also emphasised the correlation between SST and monsoons, through which the distribution of SST changes according to the monsoon season (Daud *et al*., 2019). Other previous studies by Richier *et al*. (2003) also supported the idea that the SST distribution of SCS changes with the monsoon, suggesting that the SST variation in this study is related to the monsoon season. A previous study by Hinrichs *et al*. (2013) highlights temporal variations, specifically seasonal variations of SST that impacted the health of *A. digitifera* at

Ningaloo Reef, Australia. This stress modulates the stress response of coral and increases antioxidant enzyme activities to cope with the stressor. This is also supported by Liñán-Cabello *et al*. (2009), who showed temporal changes in antioxidant enzymes whereby GST activity is higher during the summer, as indicated by high SST in *Pocillopora capitata*.

Not only SST but the effect of elevated or prolonged high temperatures on corals in general has been thoroughly studied, with the conclusion that it may affect their antioxidant enzyme activities (Lopes *et al*., 2018; Dias *et al*., 2019; da Silva Fonseca *et al*., 2021; Safuan *et al*., 2021). The fluctuation of SST in 2018 is considered normal and *A. digitifera* managed to prevent extreme production of ROS as presented by antioxidant enzyme activities, especially during May, where the GST activity increased at 30.60°C to counteract the effects of ROS. It was significantly reduced in July when the temperature dropped to 29.80°C. Constant fluctuation throughout the year with a slow rate of temperature change may also improve their tolerance in their natural habitat (Samshuri *et al*., 2023). However, under experimental conditions, *A. digitifera* may behave differently and lose its zooxanthellae at 31.00°C due to the drastic temperature change at 0.50°C per day, although the antioxidant enzyme CAT and GST were not induced (Safuan *et al*., 2021). In this study, if we consider the rate of temperature increase from the lowest temperature in January to May, it was only 0.50°C per month, which is highly lower than Safuan *et al.* (2021). Although no visible bleaching was observed during the sampling, it is important to note that the symbiotic relationship of coral in SCS greatly depends on temperature (Tong *et al*., 2017). Temperature has been known long ago to have a greater impact on the symbiosis between coral and zooxanthellae; therefore, it is suggested that zooxanthellae density is an important additional variable in future studies to understand better the biochemical behaviour of *A. digitifera* in Pulau Bidong.

Conclusions

The presence of multiple stressors can lead to an increase in the generation of reactive oxygen species (ROS), with high sea surface temperatures (SST) are particularly noteworthy in this regard, as demonstrated in the study. Corals trigger the activation of antioxidant enzymes such as catalase (CAT) and glutathione S-transferase (GST) to mitigate the impact of oxidative stress. These enzymes serve as the primary and secondary lines of defence, safeguarding the cell against oxidative stress. Consequently, GST and CAT serve as valuable biomarkers for evaluating the protective mechanisms employed by corals in response to environmental stressors. In this study, the steady level of the enzyme activities suggests a sufficient baseline level of antioxidant enzyme activities, particularly CAT, exhibited by *A. digitifera*. The SST fluctuations in 2018 were considered normal based on previous yearly trends, with no drastic temperature changes observed. By monitoring the activity of these antioxidant enzymes, future studies could gain valuable insights into how coral copes with varying environmental conditions such as temperature fluctuations. The outcomes of this work are useful for studying the long-term responses of corals and provide a deeper understanding of their adaptive strategies over a certain period. This information is crucial for developing effective conservation and management strategies to protect coral reef ecosystems in the face of ongoing environmental changes.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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