

THOMÁS NEI SOTO BANHA

Experimental effects of multiple thermal stress events on chlorophyll-a content and size of *Cassiopea andromeda* and the role of heterotrophic feeding and *Symbiodinium* concentration

Thesis presented to the Instituto Oceanográfico of the Universidade de São Paulo, in partial fulfillment of the requirements for obtaining the degree of Master in Sciences, Oceanography Program, concentration area of Biological Oceanography.

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(CORRECTED VERSION)

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“It is not wanting to win that makes you a winner.

It is refusing to fail.”

Peyton Manning

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RESUMO

O branqueamento é definido pela ruptura da relação simbiótica entre o hospedeiro e o dinoflagelado *Symbiodinium*, resultando na exposição do esqueleto de carbonato de cálcio do hospedeiro, e tem sido associado principalmente à elevação da temperatura da água do mar. Esses eventos estão se tornando mais intensos e frequentes, prejudicando recifes de corais ao redor do mundo, resultando em branqueamento de quase 100% da comunidade em alguns locais. Portanto, é importante entender se os efeitos desses eventos de estresse são cumulativos neste relacionamento fotossimbiótico. No presente estudo foi experimentalmente testado como medusas de *Cassiopea andromeda* em simbiose com *Symbiodinium* são afetadas por três eventos consecutivos de estresse térmico, monitorando o tamanho da *umbrella* e a concentração de clorofila-a. Medusas em quatro condições fisiológicas em relação à frequência de aporte heterotrófico (diário e a cada três dias) e a concentração de *Symbiodinium* (baixa e alta) foram sujeitas a um estresse térmico de uma semana em cada rodada experimental. Foram aplicados três tratamentos: 27°C (controle, mantido nesta temperatura durante todo o experimento), 30°C e 33°C. Após um período de recuperação de três semanas a 27°C, os eventos de estresse térmico foram aplicados em mais duas instâncias interligadas por um período de recuperação. *C. andromeda* branqueou e sua clorofila-a diminuiu para todas as temperaturas durante o primeiro estresse térmico e apenas a 33°C para o segundo e terceiro eventos. O tamanho só foi afetado pela alimentação e pelo segundo estresse térmico. A oferta de alimento mais frequente fez com que os organismos aumentassem de tamanho, enquanto aqueles aos quais se oferecia com uma menor frequência, diminuíram de tamanho. Conforme observado em outros hospedeiros, a alta concentração de *Symbiodinium* resultou em perda de clorofila-a, provavelmente devido à produção excessiva de espécies reativas de oxigênio e auto-sombreamento. Medusas de *C. andromeda* branquearam apenas no primeiro evento de estresse e sua resiliência a múltiplos estresses térmicos depende de condições bióticas e abióticas. Não houve diferença na mortalidade em qualquer condição ou temperatura aplicada. Sob condições de estresse térmico, o aporte heterotrófico desempenha um papel crucial no tamanho e

dependência da simbiose. A concentração de *Symbiodinium* é um fator importante nos experimentos que utilizam cenários de mudanças climáticas. Embora a concentração de simbiontes não tenha relação com o crescimento do hospedeiro, isso afeta o próprio *Symbiodinium* e, portanto, deve ser considerado em futuros experimentos. Efeitos crônicos de múltiplos estresses térmicos foram observados para a clorofila-a, enquanto estes são agudos para o crescimento. *C. andromeda* aparentemente não depende do *Symbiodinium*, especialmente sob condições estressantes e pode ser usado como modelo para investigar os efeitos das alterações climáticas no *Symbiodinium* em simbiose. Sem maior mortalidade do hospedeiro, o comportamento do *Symbiodinium* pode ser monitorado até seus limites fisiológicos após cada evento de branqueamento.

Palavras-chave: Branqueamento; Ecologia Marinha; Heterotrofia; Mudanças climáticas; Simbiose; Temperatura.

ABSTRACT

Bleaching is defined as the disruption of the symbiotic relationship between the host and *Symbiodinium* dinoflagellates, resulting in the exposure of the calcium carbonate skeleton. This phenomenon has been mostly linked to seawater temperature elevation. Bleaching events are becoming more intense and frequent, harming coral reefs around the world and affecting almost 100% of the community in some places. Therefore, it is important to understand if the effects of these recurrent stress events are cumulative on this photosymbiotic relationship. We experimentally tested how the *Symbiodinium*-associated jellyfish *Cassiopea andromeda* is affected by three consecutive thermal stress events, monitoring bell size and chlorophyll-a concentration. Jellyfishes in four physiological conditions regarding feeding frequency (daily and every three days) and *Symbiodinium* concentration (low and high) were subject to a one-week thermal stress at each experimental round. Three treatments were applied: 27°C (control, maintained in this temperature during all experiment), 30°C and 33°C. After a three-week recovery period at 27°C, thermal stress events were applied on two more instances intertwined by a recovery period. *C. andromeda* bleached and its chlorophyll-a content decreased for all temperatures during the first thermal stress and only at 33°C for the second and third events. Size was only affected by feeding and by the second thermal stress. Higher food offer caused organisms to increase in size while those offered food with lower frequency shrunk. As observed in other hosts, high *Symbiodinium* concentration resulted in loss of chlorophyll-a, probably due to excessive production of reactive oxygen species and self-shading. *C. andromeda* jellyfish bleached just in the first stress event and its resilience to multiple thermal stresses is dependent upon biotic and abiotic factors. There was no difference in mortality in any condition or temperature applied. Feeding plays a crucial role on size and symbiotic dependency under thermal stress. *Symbiodinium* concentration is an important factor in experiments testing climate change scenarios and although symbiont concentration has no relation to the growth of the host, it affects the *Symbiodinium* itself, thus might be considered in future experiments. Chronic effects of multiple thermal stresses were observed for chlorophyll-a, while these were acute for growth. *C. andromeda* apparently does

not rely only on *Symbiodinium*, especially under stressful conditions and can be used as a model to investigate the effects of climate change in *Symbiodinium* symbiosis. Without major host mortality, the behavior of *Symbiodinium* in its physiological limits after every single bleaching event can be monitored.

Keywords: Bleaching; Climate change; Heterotrophy; Marine Ecology; Symbiosis; Temperature; Upside-down jellyfish.

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LIST OF ABBREVIATIONS

AS: After Thermal Stress.

BS: Before Thermal Stress.

C: Temperature.

Chl-a: Chlorophyll a.

D: Feed Daily.

DF: Degrees of freedom.

F: Feeding.

H: High concentration of *Symbiodinium*.

HD: High *Symbiodinium* concentration and daily feeding.

HT: High *Symbiodinium* concentration and every three days feeding.

L: Low concentration of *Symbiodinium*.

LD: Low *Symbiodinium* concentration and daily feeding.

LT: Low *Symbiodinium* concentration and every three days feeding.

MRBD: Maximum Relaxed Bell diameter.

R: Recovery.

SE: Thermal Stress event.

SS: Stress Stage.

T: Feed every three days.

Z: *Symbiodinium* concentration.

1. INTRODUCTION

Coral reefs occupy 0.09% of the world's ocean area but harbor approximately 25% of the known marine species (Morrissey et al., 2018). However, this rich ecosystem is currently threatened at global and local scales. Pollution, destructive and exploitative methods of fishing and the increasing emission of greenhouse gases are some of the harmful activities currently happening (Hoegh-Guldberg, 2014). The ocean has absorbed ca. one-third of the anthropogenic CO₂, increasing almost 40% since pre-industrial levels (Sabine et al., 2004; IPCC, 2014). If emissions are not reduced, IPCC projections estimate that the average temperature of the planet will increase 2°C by 2100, with the surface layer of the oceans warming at a rate of 0.11°C per decade (IPCC, 2014).

Rise in surface seawater temperature (SST) directly affect shallow water organisms, promoting significant biochemical and ecological changes especially on reef organisms, leading to biodiversity and habitat loss within the community (Knowlton, 2001; Pandolfi et al., 2003; Kelmo et al., 2014). It is reported that temperature shifts may also cause changes in the distribution of fishes (Cheung et al., 2013), stimulate the spreading of *Vibriosis* (Vezzulli et al., 2016) and negatively impact larval development of several invertebrate taxa (Walther et al., 2010; Byrne et al., 2013). The rise in temperature can also affect coral larvae (Mies et al., 2018), reduce or impair growth and regeneration and facilitate the proliferation of diseases in corals (Meesters and Bak, 1993; Cantin et al., 2010; Francini-Filho et al., 2010; Anthony et al., 2011). This may result in a significant mortality of up to 100% of the reef population resulting in the complete devastation of reefs and reduction of biodiversity and ecosystem services (Glynn, 1983; 1984; Loya et al., 2001). Other relevant consequences include community phase-shift, which are characterized by the change of predominance from coral coverage to other taxa, especially filamentous and frondose macroalgae or sponges (Ostrander et al., 2000; Chaves-Fonnegra et al., 2017). Human populations also suffer from these negative impacts as almost 655 million people live nearby coral reefs, with ca. 300 million being dependent on their goods and services (Donner and Potere, 2007; Newton et al., 2007; Olivotto et al., 2011). One of the major benefits of coral reefs

is tourism (Brander et al., 2007). For instance, tourism related to coral reefs means annually inputs of about USD 36 billion, 9% of coastal tourism, representing USD 1657.4 million to Mexico's economy and USD 1530.7 million to USA (Spalding et al., 2017). The pharmacological industry also depends on coral, since more than 1200 new compounds extracted from marine organism can be annually discovered (Blunt et al., 2018).

The most notable impact of ocean warming on reefs is the bleaching phenomenon. Bleaching is defined as the disruption of the symbiotic relationship between *Symbiodinium* dinoflagellates (i.e., zooxanthellae, responsible for the majority of primary production in reef habitats – see Muscatine and Porter 1977; Muscatine et al. 1981) and its invertebrate host (e.g., cnidarians, foraminiferans, mollusks, sponges). Its main consequence is the loss of *Symbiodinium* from host tissue and/or degradation of photosynthetic pigments in the symbiont, which may occasionally lead to host death (Glynn, 1993; Howells et al., 2016). Theoretical and experimental frameworks show that bleaching is triggered by oxidative stress (Lesser, 1997; Weis, 2008; Krueger et al., 2015). When the holobiont is under thermal stress, both host and symbiont engage in the production of reactive oxygen species (ROS) in the mitochondria and mitochondria and chloroplast, respectively. The build-up in ROS concentration leads to cellular and DNA damage, severely compromising the holobiont as a whole (Roberty et al., 2015). In an attempt to reduce the oxidative damage, the host organism usually releases one of their major sources of ROS, its symbiont. Ultimately, this leads to the breakdown of host tissue and pigments, leaving the white calcareous skeleton visible (Swain et al., 2016)

Bleaching events have been recorded since the late 1970's, when increases in SST became critical (Hoegh-Guldberg, 1999), but this phenomenon became more intense in the 1980's. In 1983, the first mass coral bleaching event was observed in eastern Pacific and southwestern Caribbean (Glynn, 1984; Glynn, 1993; Goreau and Hayes, 1994). Its cause was later associated with both global warming and ENSO (*El Niño*–Southern Oscillation) (Glynn, 1991). The first registered global bleaching event occurred in 1998 (Goreau et al., 2000) and led to the death of 16% of world's corals (Glassom, 2014). Since then, research efforts have been focused on understanding the mechanisms, triggers, adaptations and

ecological consequences in order to mitigate impacts (West and Salm, 2003; Baker et al., 2008; Fujimura and Riegl, 2017). After the first observation, widespread mass bleaching events started to become more severe and frequent (Hughes et al., 2018). The number of impacted reefs increased threefold between 2006–12 when compared to 1985–91, with periods between events being fivefold shorter (Heron et al., 2016). Such mass bleaching events were more common in the Western Atlantic than in the Pacific and Indian Oceans and Australia (Hughes et al., 2018). From 2014 to 2017, the latter being the warmest year since 1958 (Cheng and Zhu, 2018), many reefs around the world bleached during what is considered the third global coral bleaching event, with the largest duration, area and damage ever registered (NOAA, 2015). During this event, 93% of the Great Barrier Reef corals bleached (GBRMPA, 2017) and 62% of the UNESCO's world heritage reefs, chosen for their Outstanding Universal Value, were highly impacted (Heron et al., 2017).

While still poorly monitored and with a remarkable lack of temporal data, Brazilian coral reefs encompass the vast majority of the South Atlantic reef communities. The unofficial records of bleaching events date back to the early 1990's and the first paper was published only in late 1990's (Amaral and Costa, 1998). After that, the production did not intensify until the 2010's, when almost three-quarters of the bleaching reports in Brazilian coral reefs were published (Banha et al., in prep). The identification of the bleaching phenomenon was made mostly visually while *Symbiodinium* concentration was assessed in few papers. The majority of the papers presented short timeframes or even a single observation, although some presented 8 or 17 years of monitoring (Leão et al., 2008; Kelmo and Attrill, 2013; Kelmo et al., 2014). About 90% of the events were recorded in the Northeastern region (Banha et al., in prep), probably due to its high diversity and higher number of coral reefs located within Marine Protected Areas. Bleaching was identified in 21 species, with *Siderastrea stellata* described as bleached in more than 50% of the studies, while *Montastraea cavernosa*, addressed in 13% of the works, was the most resistant among 25 species (Banha et al., in prep). The majority of the papers attributed the bleaching to temperature anomalies, varying between 0.25 and 3.2 above the average. Ultraviolet radiation,

sedimentation, low salinity and anthropic activities were also considered impacts (Barradas et al., 2010; Soares and Rabelo, 2014; Sassi et al., 2015).

While the individual resistance to bleaching is important, the resilience of organisms facing multiple thermal stress events is even more crucial since, although these events are predictable, they do not maintain the same characteristics (Trenberth, 1997; Pillai et al., 2016). The effects of multiple thermal stress events have already been investigated *in* and *ex situ* and the organisms seem to become less resistant to new stressful events (Armoza-Zvuloni et al., 2011; Middlebrook et al., 2012; Grottoli et al., 2014; Schoepf et al., 2015). The lack of resistance increases the possibility of “re-bleaching”, *i.e.* a single organism or community undergoing the negative consequences of multiple bleaching events. Only rarely the disturbance of a single variable affects coral reef communities, considering that the interaction between factors is more important than a given parameter, and can stress organisms above their physiological limits (Coles and Jokiel, 1978; Welle et al., 2017). Temperature increase is the main cause of bleaching, but other contributing factors such as water turbidity (Bessell-Browne et al., 2017; Bessell-Browne et al., 2017b; Grottoli et al., 2018), changes in salinity and available photosynthetically active radiation (Kuanui et al., 2015; Ellison et al., 2017; Bessell-Browne et al., 2017b) and reduction of pH (Anthony et al., 2008) can exacerbate its effects (Langlais et al., 2018). On the other hand, some factors may contribute to host resistance. Organisms with lower densities of symbionts may suffer less under stressful conditions and may present higher efficiency in photosynthetic output (Xu et al., 2017). Exogenous energetic inputs may also aid the host until the environmental conditions are favorable for autotrophic production. Even though some authors have reported that heterotrophic feeding is not able to compensate for either reduced photosynthetic rate or bleaching (Ezzat et al., 2013; Levas et al., 2015), several studies suggest that it can improve resilience of organisms undergoing stressful events (Clayton and Lasker, 1984; Grottoli et al., 2006; Hughes and Grottoli, 2013; Grottoli et al., 2017; Tagliafico et al., 2017; Mies et al., 2018b).

The *ex situ* manipulation and maintenance of living hermatypic corals, which are the most emblematic species when studying the effects of climate-driven temperature increase, is extremely difficult and poses a significant

challenge in performing experimental studies on bleaching for many species. However, many other easier to handle *Symbiodinium*-associated cnidarians such as anthozoans or scyphozoan polyps may be used as experimental models for this symbiotic relationship (Trench and Thinh, 1995; Pitt et al., 2005; McGill and Pomory, 2008; Howe, 2013). The use of the upside-down benthic jellyfish *Cassiopea andromeda* may also be an alternative and relevant model host organism. They present many advantages since they can be easily maintained and bred in laboratory (Hofmann et al., 1996; Leal et al., 2012). The association between *C. andromeda* and *Symbiodinium* seems to be obligatory (Rowan et al., 1997; Mellas et al., 2014; Freeman et al., 2016) and symbiont acquisition is similar to that of the majority of cnidarians (Kinzie and Chee, 1979; Lampert, 2016). In addition, they also respond to environmental stressors such as temperature variation (Clark and Jensen, 1982; McGill and Pomory, 2008). Therefore, it was decided to use this non-calcifying cnidarian as a model to test the effects of multiple thermal stress events.

The main purpose of the present work was to assess the resilience of *Cassiopea andromeda* to multiple thermal stress events. Therefore, three hypotheses were proposed:

1. *C. andromeda* bleaches proportionally less under multiple thermal stress events and bleaching is dependent of *Symbiodinium* concentration and feeding;
2. The bell diameter of *C. andromeda* is affected by multiple thermal stress events and it is dependent of *Symbiodinium* concentration and feeding;
3. Multiple thermal stress events increase the mortality of *C. andromeda*, which is independent of *Symbiodinium* and feeding.

Experimental assessments on the resilience of fauna are fundamental to understand impacts of multiple stressors in the ecology and biology of the organisms (Sparrow et al., 2017; Welle et al., 2017). In order to understand how multiple thermal stress events affects holobiont chlorophyll-a content and physiology, and what are the consequences of feeding and different concentrations of *Symbiodinium*, we simulated experimentally such conditions.

Cassiopea andromeda was selected as a model for the experiments due to its hardiness and high asexual reproduction rates (which can also be induced artificially). We aimed to determine which conditions are the more suitable or harmful to the photosymbiotic holobiont under thermal stress and investigate if the invasive *C. andromeda* can be used as a model to determine the effects of climate change on other *Symbiodinium*-associated organisms.

2. MATERIAL AND METHODS

The upside-down jellyfish *Cassiopea andromeda* was submitted to multiple thermal stress events in order to investigate their effects in chlorophyll-a content, size of bell diameter and mortality. Experiments took into consideration the time scale of the stress (acute or chronic), *Symbiodinium* concentration (high and low) and feeding (high and low feeding frequencies). Chlorophyll-a was determined by fluorometry and size of bell diameter considered the maximum relaxed length. *Symbiodinium* concentration was determined by cell counting after maceration of the ephyrae.

2.1 Molecular identification

The identities of both *Cassiopea andromeda* and *Symbiodinium microadriaticum* (type A1) had been previously confirmed through the extraction of gDNA and performing standard PCR and sequencing protocols (Sambrook et al., 1989), using the primers for internal transcribed spacer 2 (ITS2) and cytochrome c oxidase I (COI) described in LaJeunesse and Trench (2000) and Morandini et al. (2017).

2.2 Abiotic conditions

During the experiment, artificial seawater (ASW) with specific gravity of 1024 kg.m⁻³ was used to avoid enrichment with nutrients and contamination with pathogens (Fleck and Fitt, 1999). During all the experiment the organisms were kept under a photon flux of 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ and a photoperiod of 12 light: 12 dark.

2.3 Organisms

Cassiopea

Cassiopea andromeda polyps were maintained in a 56-liter round tank with an 100-liter sump, with constant water circulation and fed daily with *Artemia franciscana* nauplii (350 μm , GSL 80%, INVE Aquaculture®, hatched according to

the manufacturer's instructions). A strobilation event was induced (Figure 1 – adapted from Cabrales-Arellano et al., 2017) adding 10 μM of indomethacin in the polyp tank and more 5 μM after five days. This allowed the production of ephyrae of similar age and bell diameter.

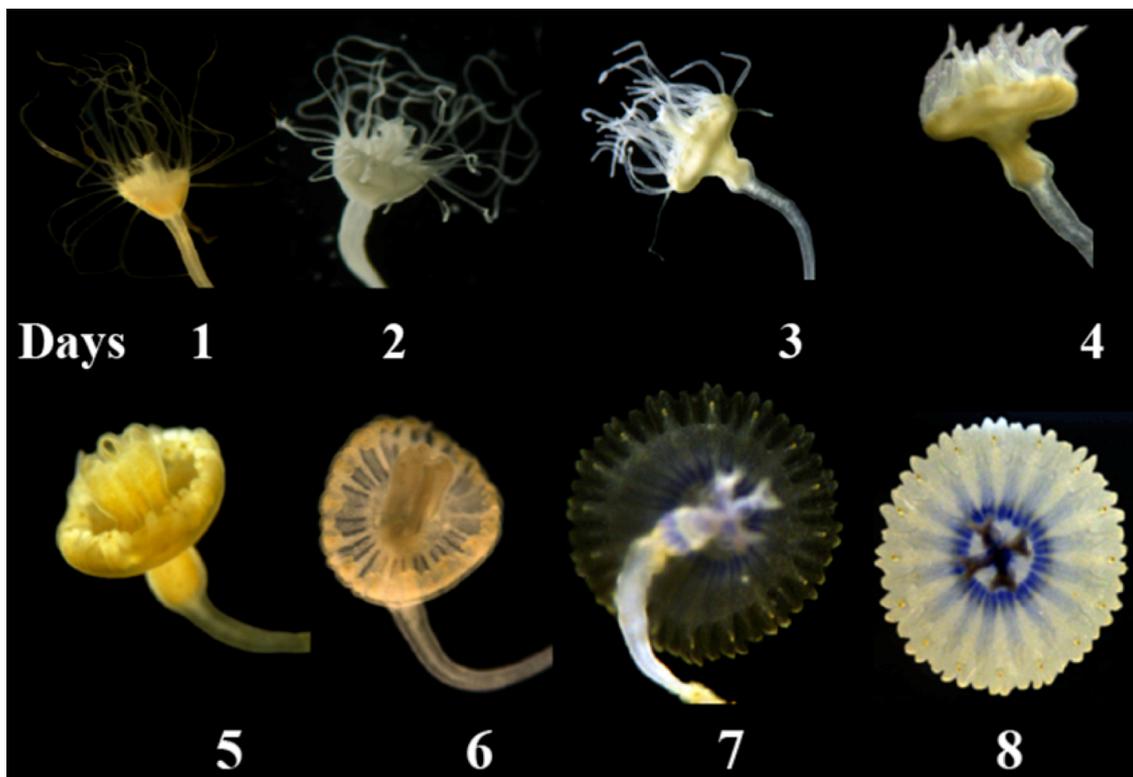


Figure 1: Example of the stimulated strobilation of polyps using indomethacin, with release of the ephyra of *Cassiopea xamachana* in day 8 (Modified from Cabrales-Arellano et al., 2017).

The ephyrae population was divided in two groups to compare the role of *Symbiodinium* concentration on jellyfish undergoing thermal stress. One group was raised together with adult jellyfish and polyps and the other grew isolated. This allowed that only one group received symbionts from the environment (i.e., horizontal transmission) shed by adults and polyps (Sachs and Wilcox, 2006; Mellas et al., 2014; Lampert, 2016). Therefore, at the end of the growth phase there was a visual difference between the two groups, one with low concentration of *Symbiodinium* (bluish coloration – Figure 2A) with average of 741.91 ± 122.23 cells. mm^{-2} and the other with high concentration of *Symbiodinium* (and brownish color – Figure 2B) with average of $3,099.39 \pm 576.45$ cells. mm^{-2} .

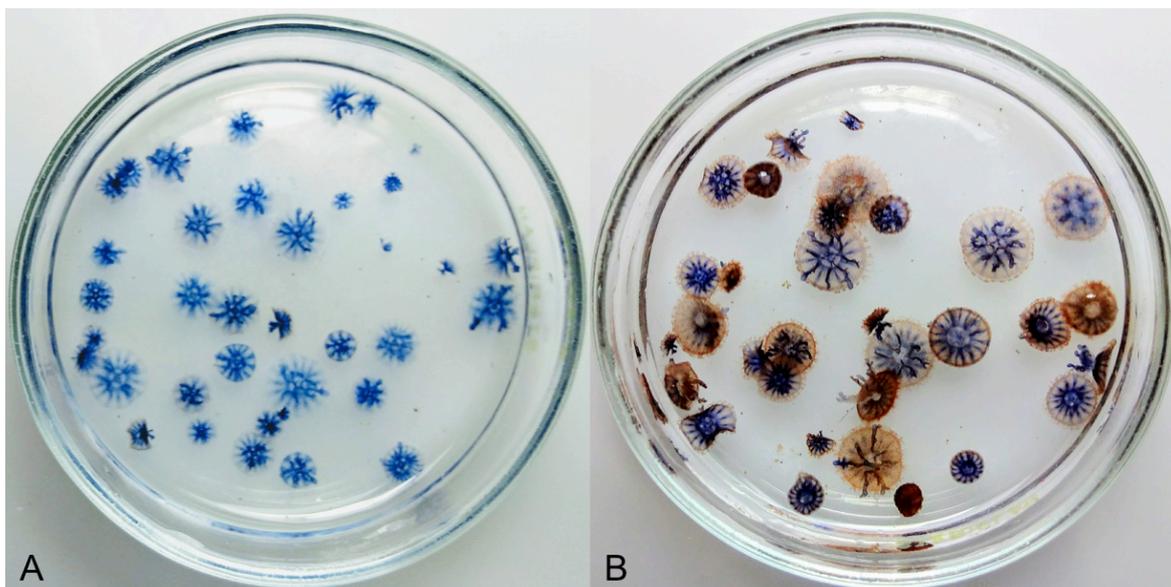


Figure 2: *Cassiopea andromeda* juveniles with (A) low and (B) high concentration of *Symbiodinium* A1 used to study the effects of thermal stress in the symbiosis of *Symbiodinium* and *C. andromeda* juveniles. Note that higher concentrations of the symbiont give the individuals a brownish coloration (B).

2.4 Experiment

The experimental setup was based on a split plot design (Figure 3), which proposes that easy-to-change factors could be manipulated within difficult-to-change factors (Altman and Krzywinski, 2015). It was selected due to the difficulty to replicate the experiment for each temperature individually, which would result in too many replicates and time spent.

We used a fully crossed design with randomized sampling and treatments consisting of three temperatures (27° – control, 30° and 33°C), two feeding frequencies (daily and every three days), two *Symbiodinium* densities in juveniles (low and high) and three replicates for each treatment combination, totaling 36 Erlenmeyer flasks (i.e., experimental units) (Figure 4). Each replica had a volume of 1120 ml and 28 juveniles, resulting in a concentration of one jellyfish per 40 ml. Each culture flask was covered to prevent excessive water evaporation and consequent changes in salinity.

The experiment took 12 weeks to be completed. There were three thermal stress events (SE1, SE2 and SE3) each lasting one week (McGill and Pomory, 2008). After each stress, cultures were allowed a 3-week recovery period (R1, R2

and R3) prior to the next stress. Samples were taken before and after each thermal stress, with the last one after a 3-week recovery period, representing the R3.

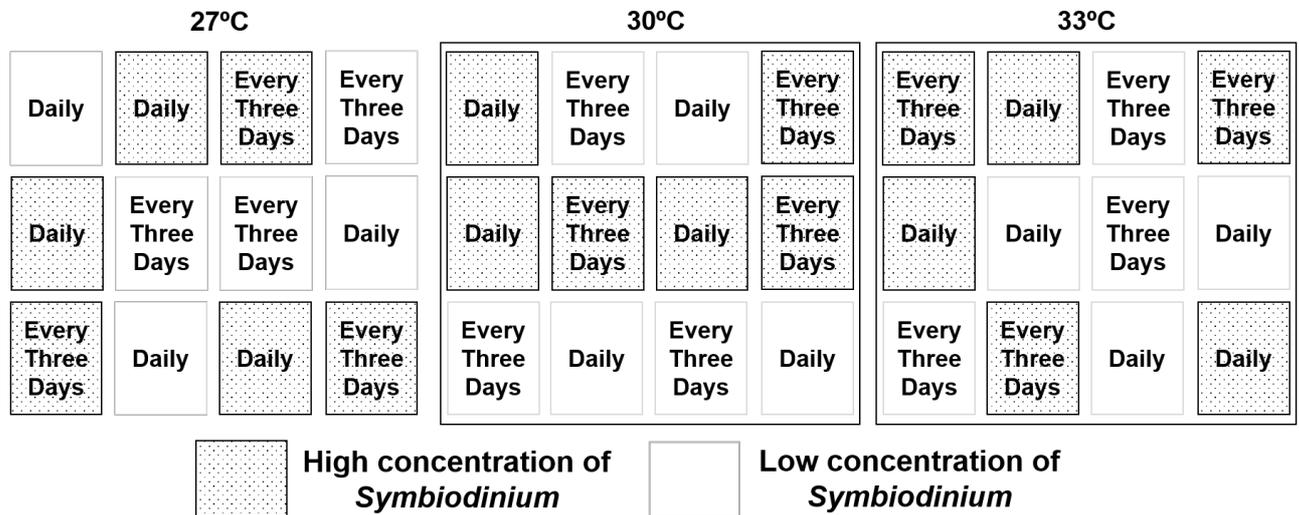


Figure 3: Experimental design to study the effects of thermal stress on the symbiosis between *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Feeding and *Symbiodinium* A1 concentration were randomized within each temperature treatment. Squares represent each juvenile culture replica.



Figure 4: Experimental set up using plastic trays and thermostats for temperature control (water baths) and Erlenmeyer flasks for experimental units to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles.

The three temperatures used in the experiment were simulated by using two 100 W Hopar H606 submersible heaters in each tray and daily monitored with a thermometer. The control temperature was 27°C (maintained constant during all the experiment), comprising the temperature in which *C. andromeda* is found in the environment (Morandini et al., 2017). The first thermal stress (30°C) was chosen because it is probably the threshold of bleaching, which can start in temperatures 1-2°C above average (Wooldrige, 2013). This temperature is predicted within the RCP8.5 scenario proposed by IPCC (2014) and may be considered a moderate thermal stress, since it may not affect some species (Hoegh-Gouldberg and Smith, 1984; Fujise et al., 2014). The second thermal stress (33°C) is considered extreme since its magnitude (i.e., +6°C in relation to normal conditions) is described as extremely harmful to organisms, including *Symbiodinium* A1 (Roberty et al., 2015), harbored by our specimens.

Two time differences between the stress stages (SS), i.e., the periods before stress (BS), after stress (AS) and recovery (R), were used to signal acute and chronic stresses. We considered the thermal stress (BS to AS, 1 week) as an acute stress and thermal stress+recovery (BS to R, 4 weeks) as chronic stress. The values of R1 are the same of BS2 and the values of R2 are the same of BS3 (Figure 5)

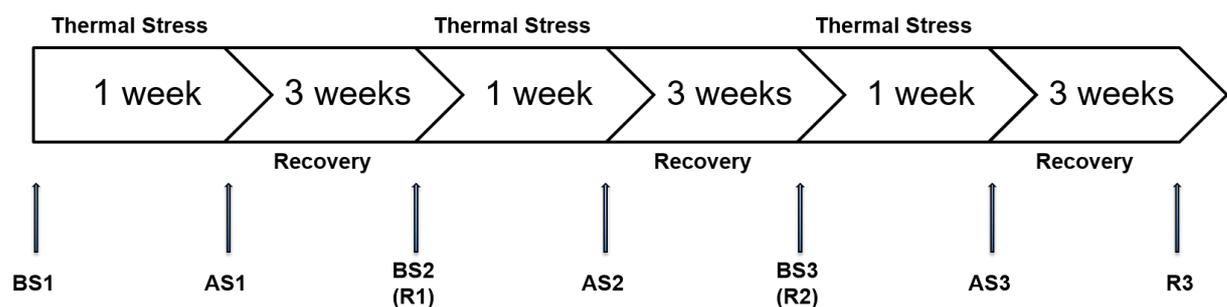


Figure 5: Timeframe of the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *C. andromeda* juveniles. The bar represents the periods of thermal stress and recovery and the overlap of some variables. BS = Before Stress; AS = After Stress; R = Recovery. Note that R1 is equal to BS2 and R2 equal to BS3.

Juveniles with a bell size diameter of 4-5 mm were chosen for the experiment in order to facilitate manipulation without damaging organisms and because of the advanced development of oral arms and easier visual identification of individuals with high and low *Symbiodinium* concentrations (Figure 2). Isolation from polyps and adult jellyfish created two groups of *C. andromeda* juveniles with different *Symbiodinium* concentrations (Z), as described before. In order to confirm visual observation, ten juveniles from each group were macerated individually and 1 ml of ASW was added. The resulting solution was homogenized and filtered using a 50- μ m mesh to eliminate the excess of *C. andromeda* tissue and the *Symbiodinium* counted in a Nageotte chamber.

Previously to the present study, we observed that *Cassiopea* with low *Symbiodinium* concentration and food deprivation presented a drastic reduction of bell diameter. Therefore, two feeding treatments (F) were adopted to test the effect of feeding on mortality and attenuation of *Symbiodinium* concentration effects on organisms and bleaching. Food with a fixed concentration of 10 *Artemia franciscana* nauplii per organism was provided daily (D) and every three days (T) for two different groups.

At each sampling, three jellyfish from each experimental unit were taken and the bell diameter measured, and then saved for chlorophyll-a (chl-a) analysis. Subsequently, two other individuals had also their bell diameter measured but returned to the culture. During measurement, organisms were removed from the culture and transferred to a Petri dish without water. This prevented jellyfish movement facilitating the maximum relaxed bell diameter (MRBD) measurements using a vernier caliper.

Sampled organisms were maintained in dark at -20°C and chl-a analysis was realized in the dark to minimize pigment degradation. Individuals were macerated with pestle and glass beads to promote cell lysis and release of chl-a. The extraction was adapted from the protocol proposed by Wasmund et al. (2006), with the addition of 10 ml of 90% acetone to the macerate. The resulting solution was kept in the dark at -20°C for 20 h. Subsequently, the content was centrifuged (3600 rpm) in the dark for 15 minutes at 10°C.

Chl-a content was read in a Turner 10AU fluorometer at 450 nm and 670 nm (Welshmeyer, 1994; Wasmund et al., 2006). Holm-Hansen et al. (1965)

equations were used to convert fluorescence values into chl-a concentrations, normalized by jellyfish area, considered as a perfect circle.

Prior to the experiment, a calibration curve was produced to assess the relation between *Symbiodinium* concentration and chl-a content. After the same maceration process described above, aliquots with estimated *Symbiodinium* cell concentrations varying from 500 to 500,000 cells had their chl-a determined. A least squares regression model was performed describe the curve.

Mortality was determined by verifying the difference of the expected number of organisms (the value that should be found before each sampling) minus the observed. In the charts of mortality, we used the modulus of the values, thus zero means that no death was registered and positive values means the number of jellyfish that died.

2.5 Experiment data analyses

To confirm the difference between the number of cells from the *Symbiodinium* concentration (Z) groups (high and low), a nonparametric Mann-Whitney's U test was performed. The relationship between *Symbiodinium* cell concentration and chlorophyll-a content was tested with a linear regression.

To investigate the effect of each of the experimental factors: temperature (C), *Symbiodinium* concentration (Z), feeding frequency (F), thermal stress event (SE) and stress stage (SS) on chlorophyll-a content and on *C. andromeda* size a 5-way crossed Analysis of Variance (ANOVA) was performed. To further compare each *Symbiodinium* concentration treatments, since the difference between high and low in chlorophyll-a content was expected, a 4-way crossed ANOVA within each *Symbiodinium* concentration was performed.

The effect of the experimental manipulation on *C. andromeda*'s mortality was assessed using four 3-way ANOVAs, for each condition studied here: high *Symbiodinium* concentration and daily feeding (HD), high *Symbiodinium* concentration and every three days feeding (HT), low *Symbiodinium* concentration and daily feeding (LD) and low *Symbiodinium* concentration and every three days feeding (LT).

In all multifactorial tests, differences between pairs (factor's levels) were tested with Student's t-test, when the pair presented more than two degrees of freedom (DF), a Tukey's HSD test was performed. In all the tests, the critical test value was 5% ($\alpha = 0.05$). To meet ANOVA assumptions of normality and homoscedasticity, data was transformed using $\log(x + 1)$ t when necessary.

3. RESULTS

The counting of *Symbiodinium* cells in the tissue of *C. andromeda* confirmed that there was a significant difference between high and low concentration groups ($U=-3.5907$; $p=0.0003$; $n=10$), which also matched the visual identification of individuals through color difference. The group visually identified as low *Symbiodinium* concentration exhibited a mean cell concentration of $20,663.75 \pm 2,832.82$ cells ml^{-1} , whereas the high concentration group had $75,555.3 \pm 9,935.9$ cells ml^{-1} . The number of *Symbiodinium* cells was positively correlated with chl-a concentration ($R^2 = 0.993$; Figure 6).

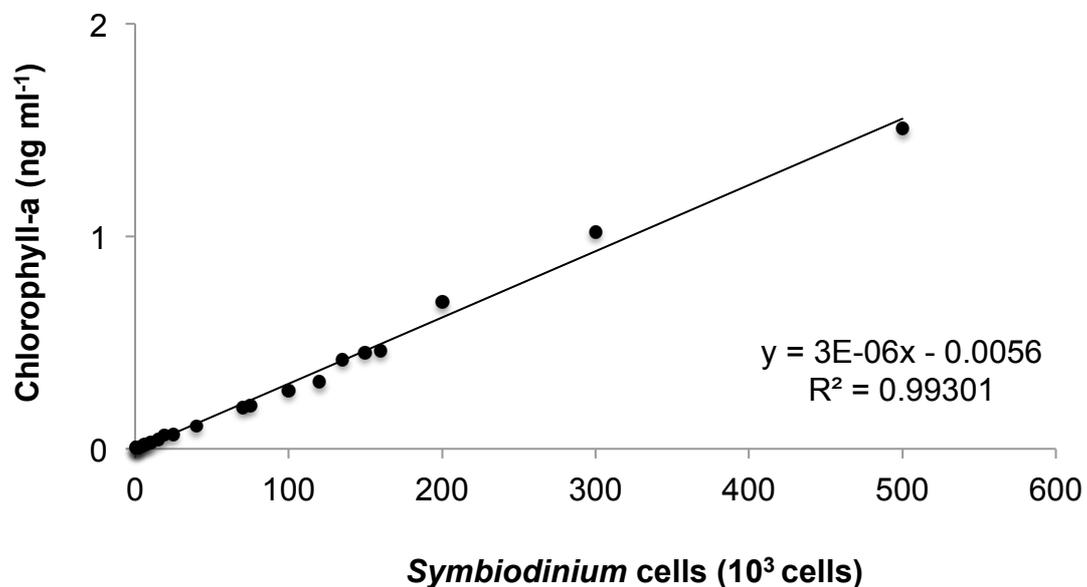


Figure 6: Linear regression between the number of *Symbiodinium* A1 cells in the tissue of *C. andromeda* juveniles and chlorophyll-a concentration measured before the experiment, showing that more *Symbiodinium* A1 cells had more chlorophyll-a.

The responses of chlorophyll-a content and size were not the same, with both having been affected differently (Table 1). There was an overall decrease in chl-a content in all temperatures in the treatment of high *Symbiodinium* concentration and daily feeding (HD) (Figure 7), with 33°C presenting the lowest final value. The same happened in the treatment of high *Symbiodinium* concentration and every three days feeding (HT) (Figure 8) at 33°C. The highest

chl-a values were found at 27°C and, together with 30°C, presented a slight increase of chl-a when comparing initial and final values.

Table 1: Results of the 5-way crossed ANOVA of size and chlorophyll-a for the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Significant values are in bold. C = temperature; Z = *Symbiodinium* concentration; F = feeding frequency; SE = thermal stress event; SS = stress stage.

Variables	Size			Chlorophyll-a	
	df	F Ratio	Prob	F Ratio	Prob
C	2	0.4227	0.6558	0.3249	0.7230
Z	1	1.5565	0.2135	786.8219	< 0.0001
CxZ	2	0.6228	0.5374	0.6112	0.5436
F	1	29.108	< 0.0001	21.3784	< 0.0001
CxF	2	0.1735	0.8408	0.3307	0.7188
ZxF	1	0.0213	0.8841	1.9657	0.1624
CxZxF	2	0.1707	0.8432	0.4033	0.6686
SE	2	11.3107	< 0.0001	10.3247	< 0.0001
CxSE	4	1.6402	0.1652	0.7011	< 0.0001
ZxSE	2	1.5099	0.2233	0.2299	0.0001
CxZxSE	4	0.2893	0.8847	0.8954	0.4676
FxSE	2	22.2193	< 0.0001	18.6732	< 0.0001
CxFxSE	4	0.6415	0.6334	0.7762	0.5417
ZxFxSE	2	0.1011	0.9039	1.1489	0.3189
CxZxFxSE	4	0.3331	0.8555	0.5584	0.6931
SS	2	20.1859	< 0.0001	28.057	< 0.0001
CxSS	4	2.9649	0.0206	2.0064	0.0947
ZxSS	2	0.1512	0.8598	10.6141	< 0.0001
CxZxSS	4	0.4587	0.7660	0.9337	0.4452
FxSS	2	16.3305	< 0.0001	18.4076	< 0.0001
CxFxSS	4	0.3208	0.8639	0.8743	0.4801
ZxFxSS	2	0.1246	0.8829	0.0857	0.9179
CxZxFxSS	4	0.3112	0.8703	0.6099	0.6559
SExSS	4	10.4564	< 0.0001	9.7860	< 0.0001
CxSExSS	8	3.7660	0.0004	1.0744	0.3821
ZxSExSS	4	1.5898	0.1781	2.9991	0.0195
CxZxSExSS	8	0.3807	0.9301	1.1067	0.3597
FxSExSS	4	5.3987	0.0004	6.2633	< 0.0001
CxFxSExSS	8	1.2541	0.2692	0.7903	0.6117
ZxFxSExSS	4	0.7891	0.5334	1.2765	0.2802
CxZxFxSExSS	8	0.4373	0.8978	0.7583	0.6399

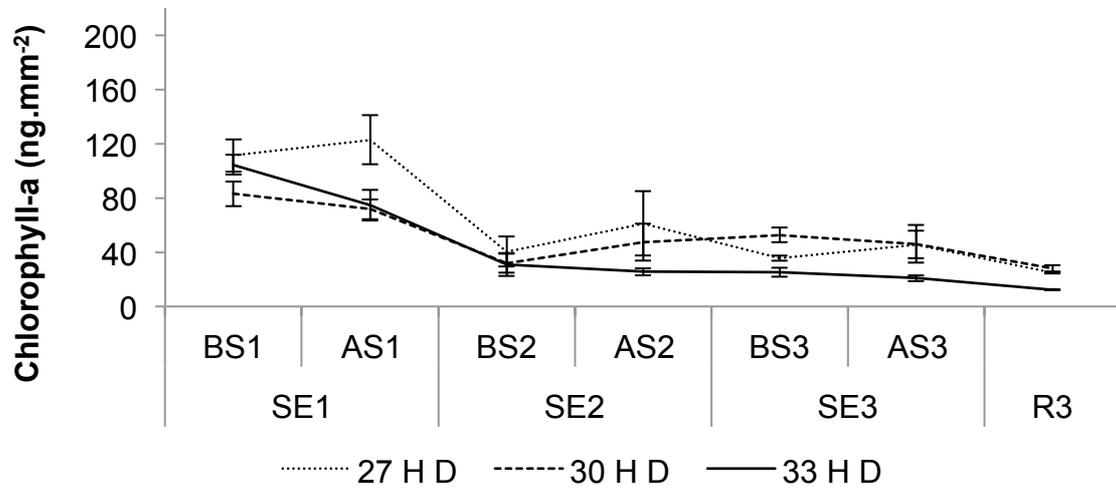


Figure 7: Chlorophyll-a content in juveniles with high *Symbiodinium* concentration and daily feeding (HD) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Chlorophyll-a is represented per area of jellyfish bell. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

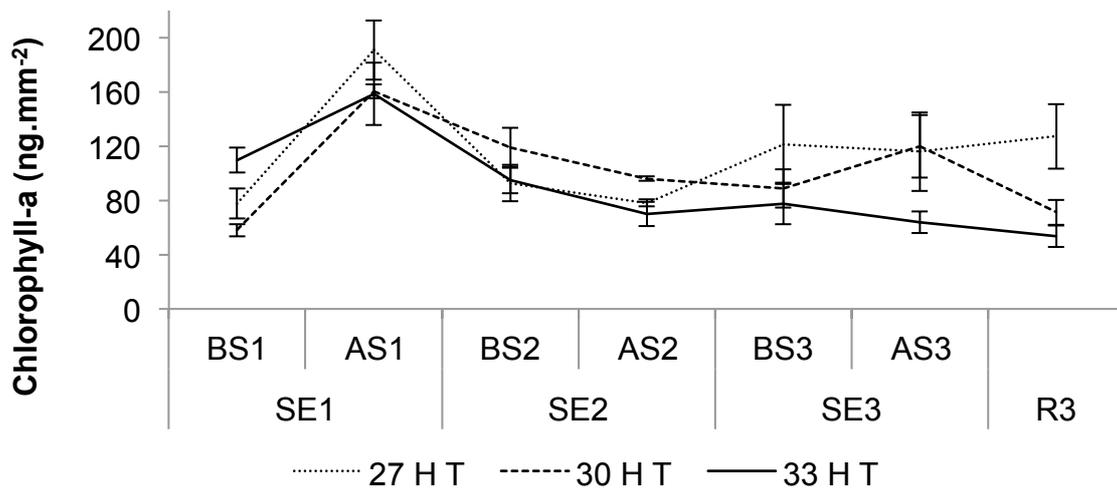


Figure 8: Chlorophyll-a concentration in juveniles of high *Symbiodinium* concentration and every three days feeding (HT) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Chlorophyll-a is represented per area of jellyfish bell. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

In low *Symbiodinium* concentration and daily feeding (LD), chl-a values were approximately similar along the experiment treatments 27 and 30°C, but decreased at 33°C (Figure 9). Overall, in of low *Symbiodinium* concentration and every three days feeding (LT), the highest increase in chl-a was observed regardless the treatment (Figure 10). The chl-a content was highest in R3 at 27 and 30°C, with values reaching 8-10-fold the initial values. This pattern was opposite for the 33°C treatment, with lower concentrations at R3.

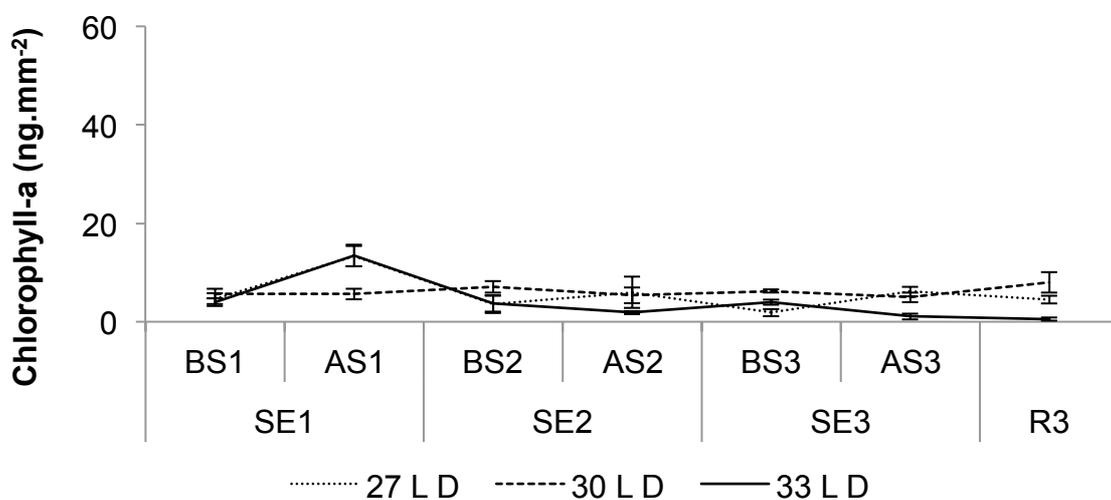


Figure 9: Chlorophyll-a concentration in juveniles of low *Symbiodinium* concentration and daily feeding (LD) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Chlorophyll-a is represented per area of jellyfish bell. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

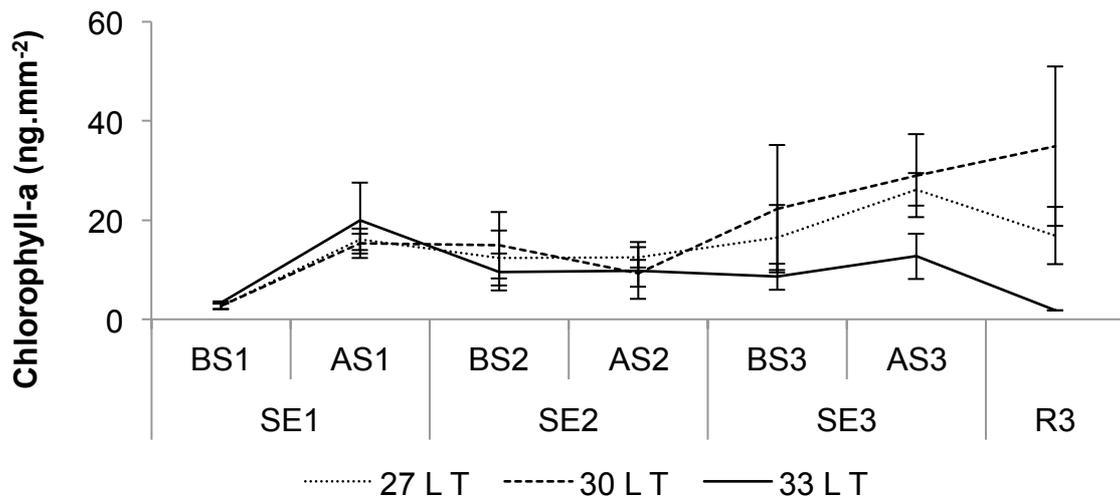


Figure 10: Chlorophyll-a concentration in juveniles of low *Symbiodinium* concentration and every three days feeding (LT) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. Chlorophyll-a is represented per area of jellyfish bell. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

There was an overall increasing pattern in bell diameter in all temperatures in high *Symbiodinium* concentration and daily feeding treatment (HD) (Figure 11). In high *Symbiodinium* concentration and every three days feeding (HT), we are able to see an opposite pattern (Figure 12), with all temperatures presenting a slight decrease.

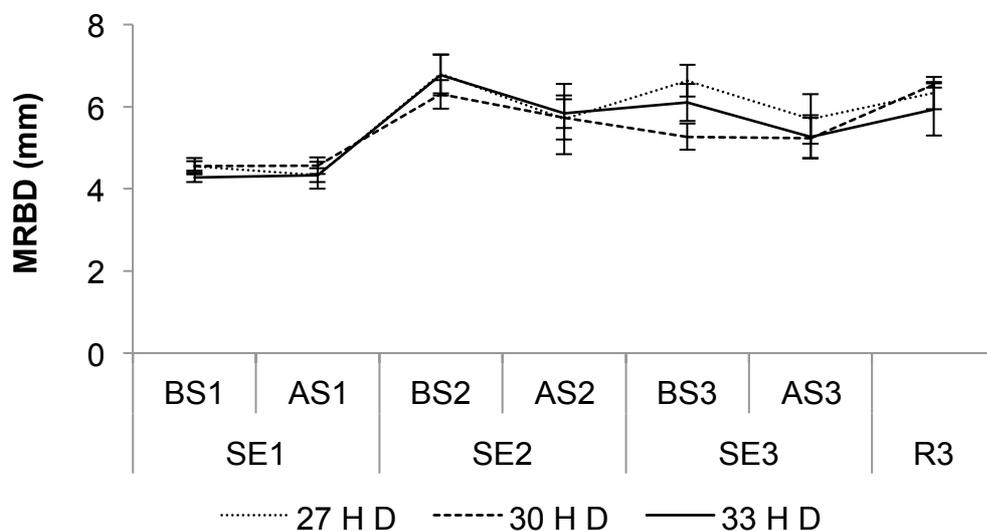


Figure 11: Changes in the maximum relaxed bell diameter (MRBD) of *Cassiopea andromeda* measured in the high *Symbiodinium* concentration and daily feeding (HD) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *C. andromeda* juveniles. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

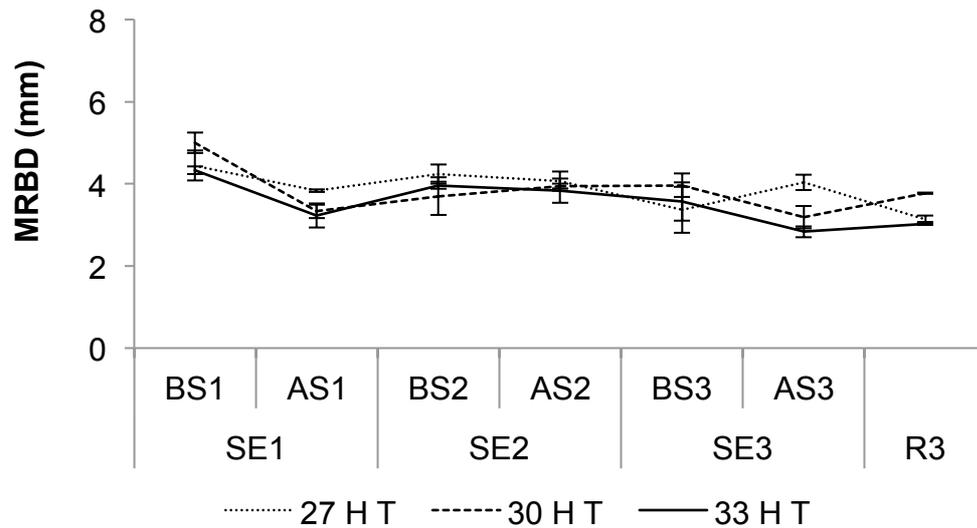


Figure 12: Changes in the maximum relaxed bell diameter (MRBD) of *Cassiopea andromeda* measured in the high *Symbiodinium* concentration and every three days feeding (HT) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *C. andromeda* juveniles. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

In low *Symbiodinium* concentration and daily feeding (LD), we observed the same pattern of high *Symbiodinium* concentration and daily feeding (HD), with an increase in bell diameter in all temperatures (Figure 13). In low *Symbiodinium* concentration and every three days feeding (LT), as observed in high *Symbiodinium* concentration and every three days feeding (HT), there was a slight decrease in bell diameter in all temperatures (Figure 14).

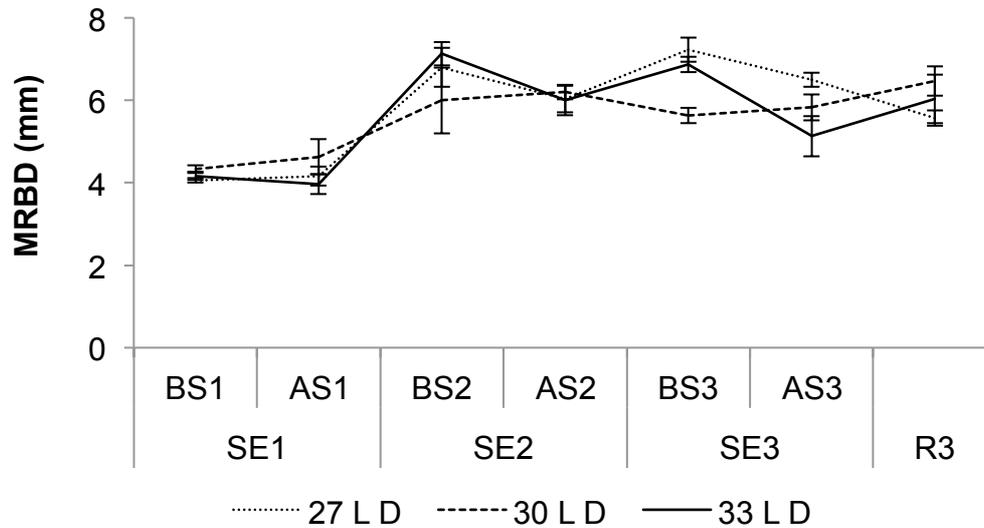


Figure 13: Changes in the maximum relaxed bell diameter (MRBD) of *Cassiopea andromeda* measured in the low *Symbiodinium* concentration and daily feeding (LD) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *C. andromeda* juveniles. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

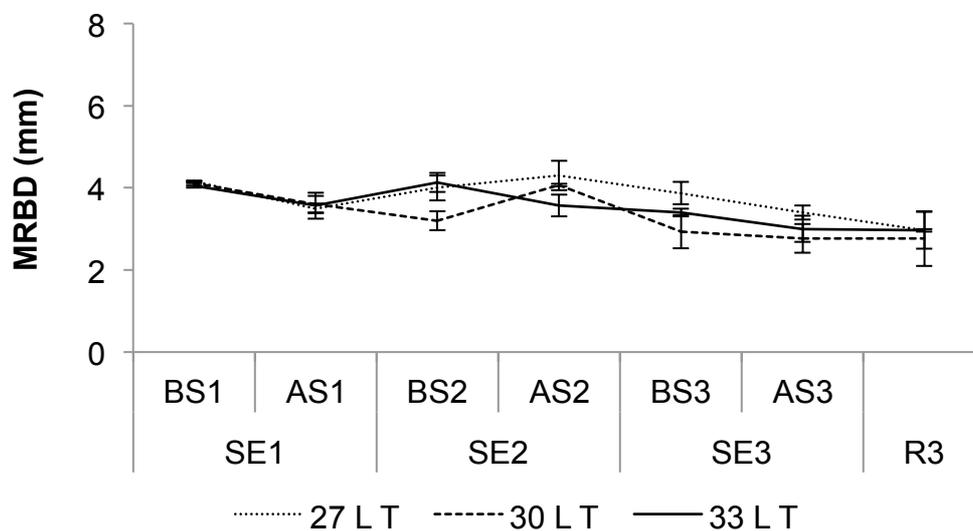


Figure 14: Changes in the maximum relaxed bell diameter (MRBD) of *Cassiopea andromeda* measured in the low *Symbiodinium* concentration and every three days feeding (LT) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *C. andromeda* juveniles. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

Temperature was significant for chl-a depending on the thermal stress event (5-way crossed ANOVA, $F_{\text{SEX}}=9.7011$, $p<0.0001$, $DF=4$), but with no clear pattern. For size, temperature was significant depending on the stress stage (5-way crossed ANOVA, $F_{\text{CxSS}}=2.9649$, $p=0.0206$, $DF=4$), with the higher sizes after recover and a decrease right after the thermal stress ($Q=3.12908$, $p<0.05$). The effect of the stress on size, which depended on stress stage, depended on the thermal stress event (5-way crossed ANOVA, $F_{\text{CxSExSS}}=3.7660$, $p=0.0004$, $DF=4$), but without a clear pattern.

Following the visual and symbiont concentration difference, there was difference in chl-a of the two groups (5-way crossed ANOVA, $F_z=786.8219$, $p<0.0001$, $DF=1$), with organisms with high *Symbiodinium* concentration having more chl-a ($t=1.96946$, $p<0.05$). The chl-a content also depended on which thermal stress event the *Symbiodinium* concentration is observed (5-way crossed ANOVA, $F_{z \times \text{SE}}=9.2299$, $p=0.0001$, $DF=2$), with a continuous decrease of chl-a in high *Symbiodinium* concentration and a decrease followed by an increase in low *Symbiodinium* concentration ($Q=2.87187$, $p<0.05$). The chl-a content of the *Symbiodinium* concentrations also depended on the stress stage (5-way crossed ANOVA, $F_{z \times \text{SS}}=10.6141$, $p<0.0001$, $DF=2$). Finally, there was an interdependence of which stress stage we look within the thermal stress event (5-way crossed ANOVA, $F_{z \times \text{SE} \times \text{SS}}=2.9991$, $p=0.0195$, $DF=4$), but again with no clear pattern. As expected, organisms with high *Symbiodinium* concentration always had more chl-a than organisms with low concentration of *Symbiodinium* ($t=1.96946$, $p<0.05$). When analyzing concentrations separately (High and Low), organisms with more symbionts were influenced by more variables and interdependences. Thus, the variation of chl-a content really depends on the symbionts concentration. In relation to size, the *Symbiodinium* concentration was not significant in any condition.

Feeding affected chl-a (5-way crossed ANOVA, $F_F=21.3784$, $p<0.0001$, $DF=1$) and organisms fed every three days had more chl-a than organisms fed daily ($t=1.96946$, $p<0.05$). The effect depended on the thermal stress event (5-way crossed ANOVA, $F_{F \times \text{SE}}=18.6732$, $p<0.0001$, $DF=2$), with no difference of the effects on organisms fed every three days and a decrease in organisms fed daily

($Q=2.87187$, $p<0.05$). The effects of feeding on chl-a also depended on stress stage (5-way crossed ANOVA, $F_{F \times SS}=18.4076$, $p<0.0001$, $DF=2$), but with no clear pattern. The effects of feeding on chl-a were different depending on stress stage and thermal stress event together (5-way crossed ANOVA, $F_{F \times SE \times SS}=6.2633$, $p<0.0001$, $DF=4$), again with no pattern.

The feeding scheme had a significant effect on the size of the organisms (5-way crossed ANOVA, $F_F=29.1080$, $p<0.0001$, $DF=1$), with jellyfish fed daily having higher diameter than those fed every three days ($t=196946$, $p<0.05$). The effect in size of the ephyrae also depended on the thermal stress event (5-way crossed ANOVA, $F_{F \times SE}=22.2193$, $p<0.0001$, $DF=2$) and stress stage (5-way crossed ANOVA, $F_{F \times SS}=16.3305$, $p<0.0001$, $DF=2$). The effects of feeding on stress stage depended on the thermal stress event (5-way crossed ANOVA, $F_{F \times SE \times SS}=5.3987$, $p=0.0004$, $DF=4$). Organisms fed daily grew more than organisms fed every three days ($t=1.96946$, $p<0.05$)

There were no differences in mortality, which was low in all temperatures and studied conditions (Figure 15). The only major mortality event occurred in the high *Symbiodinium* concentration and fed every three days treatment (HT) at 30°C when 10 individuals died after recovery 1. As it was an outlier, it was removed from the analysis and charts.

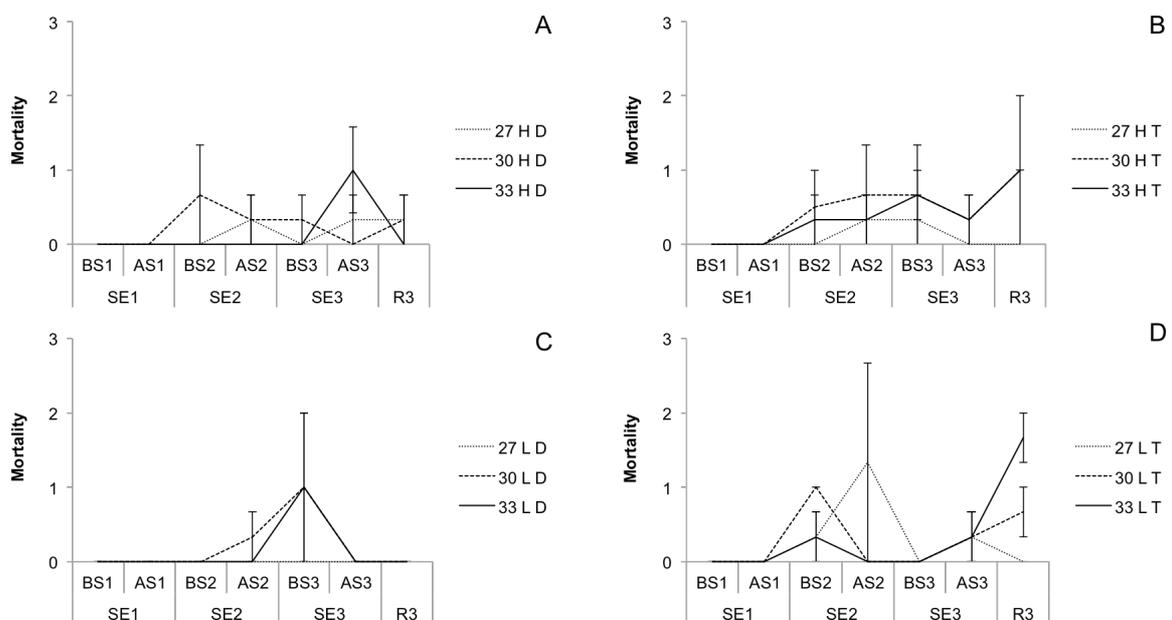


Figure 15: Mortality of organisms measured in the (A) high *Symbiodinium* concentration and daily feeding (HD), (B) high *Symbiodinium* concentration and every three days feeding (HT), (C) low *Symbiodinium*

concentration and daily feeding (LD) and (D) low *Symbiodinium* concentration and every three days feeding (LT) treatment during the experiment to study the effects of thermal stress in the symbiosis of *Symbiodinium* A1 and *Cassiopea andromeda* juveniles. BS = Before Stress; AS = After Stress; TS = Temperature Stress; R3 = Recovery 3. Error bars represent \pm one standard error.

Temperature, stress stage and thermal stress event did not significantly affect mortality in high *Symbiodinium* concentration and daily feeding treatment (HD) (3-way crossed ANOVA, $F_{HD}=0.9658$, $p=0.5250$, $DF=26$), high *Symbiodinium* concentration and every three days feeding treatment (HT) (3-way crossed ANOVA, $F_{HT}=0.6627$, $p=0.8713$, $DF=26$) and low *Symbiodinium* concentration and daily feeding treatment (LD) (3-way crossed ANOVA, $F_{LD}=0.8628$, $p=0.6521$, $DF=26$). Only in low *Symbiodinium* concentration and every three days feeding treatment (LT – Figure 15D) there was a significant difference in mortality within temperatures (3-way crossed ANOVA, $F_{LT}=2.0215$, $p=0.0147$, $DF=26$), which was dependent on the stress stage within thermal stress events is observed (3-way crossed ANOVA, $F_{SS \times SE}=4.2000$, $p=0.0049$, $DF=4$). It was caused by the mortality difference within temperatures, in different times, especially in recovery 3 (R3). Although different, no pattern was identified by the *post hoc* test ($t=2.00488$, $p<0.05$), and consequently no single explanation other than a randomly observed natural mortality.

There was a difference between the effects of stress stages on chlorophyll-a concentration (5-way crossed ANOVA, $F_{SS}=28.0570$, $p<0.0001$, $DF=2$). Chl-a contents before stress were the same as after recovery and values after stress were higher than both. After an increase during the thermal stress event, there was a decrease of chl-a concentration in the organisms during recovery periods. Thus, the thermal stress effect is probably chronic for chl-a. There was also a difference between stress stages in the size of organisms (5-way crossed ANOVA, $F_{SS}=20.1859$, $p<0.0001$, $DF=2$). Sizes in all the stress stages were different ($Q=2.35777$, $p<0.05$). After recovery, the sizes were highest, followed by the size before the stress and the values after stress. Thus, the effect of multiple thermal stresses on size is probably acute.

There was a difference of chl-a concentration at different thermal stress events (5-way crossed ANOVA, $F_{SE}=10.3247$, $p<0.0001$, $DF=2$). Values measured in the first thermal stress event were higher than those measured in the second

and third thermal stress event ($Q=2.35777$, $p<0.05$), with the last two being were similar. There was also a difference in size among thermal stress events (5-way crossed ANOVA, $F_F=11.3107$, $p<0.0001$, $DF=2$). Jellyfish measured in the second thermal stress event were larger than in the first and third thermal stress events ($Q=2.35777$, $p<0.05$)., with the last two not statistically different. Thus, the first thermal stress damaged the symbiont, but it was not affect by the second and third thermal stress events. Yet, the size of the organisms increased in the second thermal stress event and then decreased, returning to sizes near to the initial observed.

4. DISCUSSION

The hypotheses presented in this study state that (a) chl-a content, (b) size and (c) mortality in *Cassiopea andromeda* are affected by multiple thermal stress events and that these factors depend on either *Symbiodinium* concentration or frequency in food offer. As previously shown in the results, chl-a content depended on all variables and the jellyfish bleached under certain conditions, but only in the first stress event, without re-bleaching. Size depended only on feeding. Mortality was not affected by any variable. Therefore, the null hypothesis could be rejected for mortality and only partially rejected for chlorophyll and size. The calibration curve showed that relationship of chl-a and *Symbiodinium* concentration is positive and maybe it is a good proxy to determine the health of holobiont. If a calibration curve is made for healthy organisms and posteriorly made with organisms of unknown health status, variations in the values of chl-a or *Symbiodinium* concentration in the linear regression could mean that the specimen underwent stress.

The temperature tolerance threshold of marine organisms is extremely variable and species-specific and this can affect the chlorophyll production. Data produced in this work showed that 27 and 30°C had similar non-harmful effects, whereas organisms subjected to 33°C had a decrease in chl-a content. Authors found that *Cassiopea* sp. polyps can survive but not thrive at 28°C (Klein et al., 2016), while *C. andromeda* jellyfish was already found living in areas with temperatures $\geq 30^\circ\text{C}$ (Çevik et al., 2006; Özgür and Öztürk, 2008). Thus, resistance is variable within the genus. It is known that temperature increases of 1°C (Amaral et al., 2006; Wooldrige, 2013) or even 0.25°C (Kikuchi et al., 2010) above average can lead to bleaching in some species. At the same time, some corals considered extremely resistant bleach only in temperatures $\geq 6^\circ\text{C}$ above summer average and during longer periods (Grottoli et al., 2017). Corals like *Stylophora pistillata* and *Seriatopora hystrix* bleach after only a 7-hour exposure to water temperatures $> 30^\circ\text{C}$ but not under 30°C (Hoegh-Guldberg and Smith, 1984), which is the same temperature value that impairs photosynthetic efficiency in *Symbiodinium* (Iglesias-Prieto et al., 1992). Therefore, it is likely that the

photosymbiotic holobiont used as a model may not resist to 33°C, independent of the condition they are found.

Similarly to chlorophyll-a, the impact of temperature rise in size vary among species and depends on the magnitude of the increase. Here, there was no difference in the effect of temperature in bell diameter, just the increase in size related to daily feeding. Increases in temperature (within physiological boundaries) and food availability promoted exponential (i.e. faster) growth in *Aurelia aurita* and *Pelagia noctiluca* (Lucas, 2001; Widmer, 2005; Rosa et al., 2013), which partially agree with the results of this work. Temperature increase above the limits caused reduction in size on *Cassiopea xamachana* (McGill and Pomory, 2008), which also agrees in part with our results. Even though there was no difference among temperatures, *C. andromeda* fed every three days (same feeding frequency adopted by McGill and Pomory, 2008) also had its bell diameter reduced. A temperature of $\geq 1.5^{\circ}\text{C}$ above the ideal affected *Aurelia labiata* ephyrae, impairing growth, feeding and even swimming (Widmer, 2005), with the latter two consequences, caused by eversion of the bell, condition observed in this work (pers. obs.) and, thus, probably also affecting *C. andromeda*.

Higher *Symbiodinium* concentration usually means higher chl-a content, which can be observed in the calibration curve (Figure 6) and in the difference between the initial values from high and low treatments here used (Figures 8-11). However, during the experiment, daily fed organisms with high concentration of *Symbiodinium* had a constant decrease in chl-a. Authors observed that reef corals with higher symbiont density appear to be more vulnerable to climate change-induced effects (Nesa and Hidaka, 2009; Cunning and Baker, 2013; 2014) and, in this case, the symbiosis benefits decrease (Wooldridge, 2016). Reductions in chl-a content were also found for *Acropora aspera* after suffering heat stress (Uddin, 2015). Organisms fed every three days with low *Symbiodinium* concentration had the highest increase in chl-a. These findings agree with the studies above and with Xu et al. (2017), who found that the efficiency of photosystem II is higher in reef corals with low *Symbiodinium* concentration. It is likely that with more symbionts being stressed, more ROS is produced within the host tissue, especially if symbionts are more susceptible to photodamage (Cunning and Baker, 2013), since different clades of *Symbiodinium* have different thermal tolerances (Rowan,

2004). This seems to be true for *C. andromeda*, since they harbor *Symbiodinium* clade A1 which, although was considered to have a high degree of thermal tolerance (Robison and Warner, 2006), is not among the most thermally-tolerant strains (Swain et al., 2017). Alternatively, self-shading may play a role as high symbiont concentration may inhibit the photosynthesis of organisms located in deeper tissue layers. As a response, these organisms may naturally reduce their symbiont population (Cunning et al., 2015).

Although fed corals had higher symbiont concentrations and chl-a content (Grottoli, 2002; Rodolfo-Metalpa et al., 2008), zooxanthellate cnidarians seem to be preferably autotrophic and may adopt a higher heterotrophic contribution just under stressful condition (Ezzat et al., 2013; Mies et al., 2018b). However, the role of heterotrophy to prevent bleaching or compensate a reduction of primary production is species-dependent (Treignier et al., 2008; Ezzat et al., 2013). In the present study, the chl-a content of the holobiont decreased with higher food offer and vice-versa. The same result was found for *Astrangia poculata* (= *A. danae*), which lost most of their *Symbiodinium* and had high pheophytin content within their tissue at higher feeding frequency (Szmant-Froelich and Pilson, 1980). Additionally, when eating less frequently, *A. poculata* did not lose symbionts (Szmant-Froelich and Pilson, 1980). These authors hypothesize that with higher food content to digest, the higher metabolism promotes an unhealthy intracellular environment for *Symbiodinium*. *Astrangia poculata* can also live almost without symbionts and seems to allow increases in *Symbiodinium* concentration just when symbiosis is advantageous (Dimond and Carrington, 2008). So, although this partnership can be considered fundamental and extremely important for scleractinian corals (Houlbrèque and Ferrier-Pagès, 2009; Tornabene et al., 2017), the symbiosis with *Symbiodinium* is facultative for some species. In *C. xamachana* holobiont, despite using heterotrophic feeding as a nitrogen source, *Symbiodinium*-carbon assimilation can be six times greater than those found in sponges or octocorals also harboring photosymbionts (Freeman et al., 2016). Therefore, it can even be considered that this organism functions as an autotroph by some authors (Freeman et al. 2016), forming an obligatory symbiosis like that of hermatypic corals (Rowan et al., 1997; Mellas et al., 2014). However, *C. andromeda* under stressful conditions may not rely on *Symbiodinium* when it has

plenty of food available, rendering its relationship with *Symbiodinium* perhaps not obligatory, although there are no records of individuals without symbionts (Stoner et al., 2016). Smaller individuals of *Cassiopea andromeda* rely more on the energy provided by symbionts than larger adults (Szu-Chien, 2010). The same was observed here, where the smallest organisms presented the highest chl-a content. Therefore, our organisms with lower feeding frequency, especially the ones with also lower *Symbiodinium* concentration, probably rely on *Symbiodinium* as energy source.

The slower growth of organisms that received less food indicates that extra heterotrophic source of energy is important for *C. andromeda*. This effect is well known among cnidarians, since feeding can promote a many fold increase in tissue growth (see Ferrier-Pagès et al., 2003), mainly if combined with higher temperatures (Lucas, 2001; Widmer, 2005; Rosa et al., 2013). For instance, temperature is the main factor affecting *Cladocora caespitosa* growth (Rodolfo-Metalpa et al., 2008). In the present work, jellyfish size was only related to feeding frequency. McGill and Pomory (2008) observed that *C. xamachana* continuously reduced its wet weight and overall size through 11 weeks after a thermal stress, similarly to what was observed in jellyfish fed every three days, same feeding frequency of McGill and Pomory (2008). However, the same did not happen in daily fed organisms, which presented an increase of bell diameter. Thus, probably a more frequent food offer can also lead to size increase and attenuate or even mitigate the effects of bleaching, as observed to other species (Hughes and Grottoli, 2013; Tagliafico et al., 2017).

In this work, the effect of thermal stresses on chl-a was felt only immediately after the thermal stress, so can be considered chronic. Conversely, Hoegh-Guldberg and Smith (1984) observed that *Stylophora pistillata* and *Seriatopora hystrix* suffered an acute stress. The organisms studied in the present work slightly reduced in size after the thermal stress and only resumed growth on the recovery phase. These findings disagree with Al-Jbour et al. (2017) and McGill and Pomory (2008) who found that *Cassiopea* sp. do not grow and can even shrink after a chronic stress. Bozec and Mumby (2015) found that acute stresses could possibly lead to reduction on coral cover such as what happens in chronic effects. However, under chronic conditions, corals have higher resilience. Chronic

effects on mortality of young organisms were also found by Bozec and Mumby (2015). This could explain the extreme mortality event that happened in one of the treatments of the present work. Schoepf et al. (2015) found that multiple bleaching events diminished the capacity of recovery of one Caribbean hermatypic coral species, while other two could recover after bleaching. The authors mention that energy reserves are necessary for resistance when facing bleaching events. Here, resilience and/or recovery also depended on the condition of organisms. This finding agrees with Grottoli et al. (2017) who also found that some characteristics are necessary for the hermatypic coral resistance to stress, such as high baseline heterotrophic feeding and energy reserves. However, it is curious that, in the present work, the highest proportional increase in chl-a occurred in the group with less symbionts and fed every three days. This shows that when *Symbiodinium* is the main energy source, organisms probably maintain the symbionts even if they can be harmful.

The association *Symbiodinium-Cassiopea andromeda* studied here presents biological responses to thermal stress that are also observed in hermatypic corals and other organisms used as models for climate change effects (Robison and Warner, 2006; Suggett et al., 2008; Jiang et al., 2017). The effects of temperature, *Symbiodinium* concentration, feeding and multiple stresses on the photosymbiotic holobiont have already been observed in reef corals. It is important to note that the host part does not seem to be affected by these stress events, since *Symbiodinium* concentration did not affect size and organisms fed with higher frequency could indeed increase in size. Considering that *C. andromeda* inhabits the very shallow waters of mangroves, saltmarshes and sea grass banks (Freeman et al., 2016; Gedan et al., 2017), it is likely that it may be more often submitted to stressful conditions of temperature and light, resulting in higher tolerance to environmental changes. The responses of *Symbiodinium* within the non-calcifying invertebrate used here were very similar to what is described in the literature when associated with hermatypic corals. *C. andromeda* possesses asexual reproduction that can be artificially stimulated (Pierce, 2005; Cabrales-Arellano et al., 2017) resulting, as observed here, in organisms with almost the same size and age, which together with the findings of this work, renders *Cassiopea andromeda* a good model to test the effects of climate change.

5. CONCLUSIONS

In conclusion, our findings suggest that the symbiont resists to multiple thermal stresses in the scenario of 30°C and just continuously bleaches under 33°C. The initial density of symbionts is relevant to the bleaching of the holobiont, especially at 33°C. Heterotrophy plays a fundamental role and seems to compensate the possible damage from multiple thermal stresses in *Cassiopea andromeda*. The effects of stresses are chronic to the symbiont and acute to the host. Finally, the resistance of the host to multiple thermal stresses is indirectly threatened by future rises in temperature. We suggest that future works should investigate the food availability and symbionts density of *C. andromeda* in the environment to couple with our results to make predictions of how *C. andromeda* will respond to environmental changes.

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