Determining Recovery Success in *Anthelia* sp. After Exposure to Varying Levels of Thermal Stress

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1. Abstract

Coral bleaching is a phenomenon caused by anthropogenically increased ocean temperatures, and may lead to the eventual death of massive reef systems. Bleaching is the result of corals expelling dinoflagellate endosymbionts in order to compensate for thermal stress. However, the loss of symbionts leads to a subsequent reduction in fluorescence intensity emitted by the coral. Substantial research has been done on coral bleaching due to environmental stressors, but little knowledge has been acquired about coral recovery after thermal stress. The present study aimed to determine how *Anthelia* species recover after being exposed to varying levels of temperature stress. Corals were exposed to varying levels of heat stress and subsequently brought back down in temperature to promote recovery. Using fluorescence microscopy, a relatively new method of quantifying coral health, and health-colour indices, recovery ability after thermal stress was determined. Analyses concluded that corals were able to successfully recover after thermal stress of 31°C, and exhibit a thermal compensation point around 30°C. However, beyond 31°C, recovery was not achievable. The findings of this study are beneficial to the larger coral research field because they indicate that corals do possess recovery ability up until reaching a fatal thermal maximum.

Keywords: Coral Bleaching, Coral Recovery, Climate Change, Coral Fluorescence, Dinoflagellate

2. Introduction

Coral reefs are one of most complex and biodiverse communities on Earth yet are extremely vulnerable to environmental changes (Roth and Deheyn 2013, Ellison et al. 2017). Numerous anthropogenic factors such as overfishing, pollution, elevated carbon dioxide exposure, and increased ocean temperatures have been shown to reduce coral health, and lead to eventual death of coral systems (Brown 1997, McClanahan et al. 2007). Unfortunately, a large portion the world's coral species have been negatively impacted by rising ocean temperatures. As such, numerous mass bleaching episodes have occurred world-wide, wherein 19% of the world's coral reefs have undergone bleaching, and are expected to continue with increasing global temperatures (Wilkinson 2008, Burke et al. 2011). However, these impacts extend beyond the coral and impact thousands of other associated species who rely on coral reefs to survive (Graham et al. 2007, Pratchett et al. 2008).

Normally, coral reef systems boast extreme biodiversity and brilliant colour schemes, which are a by-product of a symbiotic relationship between Dinoflagellate endosymbionts and coral hosts (Ainsworth et al. 2006, Oswald et al. 2007, Fujise et al. 2014a). Dinoflagellates are an autotrophic zooxanthellae species that live inside coral gastrodermis cells and provide photosynthetic products to the coral host. In return, the coral provides shelter and other nutrients for the symbionts (Muscatine and Cernichiari 1969, Hoegh-Guldberg 1999, Fujise et al. 2014a). Together, coral and dinoflagellates work in synchrony to thrive in the marine environment, while creating a foundation for entire reef communities (Wilson et al. 2010, Coker et al. 2014).

Both coral and dinoflagellates possess fluorescent proteins which are speculated to have protective properties against harmful solar radiation (Salih et al. 2000; Dove and Ranganathan 2006; Roth et al. 2010). Numerous coral species produce a fluorescent protein called pocilloporin which fluoresces in the green spectrum, whereas dinoflagellates contain

chlorophyll-a that fluoresces red and allows for efficient photosynthesis (Carpenter et al. 1991, Bou-Abdallah et al. 2006). However, when exposed to increased temperatures, dinoflagellates lose photosynthetic capability, and are expelled by the coral host (Brown 1997). Loss of symbionts, whom are essential for the coral's metabolic needs, lead to decreased health and bleaching of the coral (Fujise et al. 2014b). As coral health declines, fluorescence intensity of the coral similarly decreases (Santos and Shaw n.d.).

Substantial research and funding has been devoted to conserving coral species affected by thermal stress (Rinkevich 2005). However, little emphasis has been placed on the recovery capability of coral species after the removal of thermal stress. As such, the specific research objective of this study was to determine to what extent corals can recover to baseline health levels after being exposed to varying levels of thermal stress. It was hypothesized that if corals were able to successfully recover after removal of thermal stress, then there should be decreased recovery capability in corals stressed to higher temperatures. It was predicted that complete recovery would be exhibited by corals stressed to 29°C and 30°C, and 31°C but corals stressed to 32°C would be unable to recover. These predictions are based upon a previous study wherein 32°C (highest thermal stress) was shown to be a fatal temperature for *Anthelia* sp. (Santos and Shaw n.d.). The significance of coral recovery is important in determining how coral species can cope with the globally increasing ocean temperatures.

3. Materials and Methods

3.0 Tank Set-Up & Husbandry

To determine recovery ability of *Anthelia* corals, four thermal trials and one control trial were completed. A total of five 28.8L tanks filled with saltwater of a specific gravity of 1.025ppt were utilized in these trials. Individual samples from a larger colony in MacEwan University's main marine tank were established within each tank over months previous (n=36 samples per

tank). Fragments of corals were created by using forceps and a razor blade to sever the basal mat of the coral and separate them into smaller units. After fragmentation, corals were placed into a tank to settle onto cement plugs and rock pieces. Fragments were allowed to settle in the tank for a minimum of two weeks before experimental trials began. The purpose of an acclimation period was to allow for corals to adjustment to the new tank environment and to overcome any stress caused by the fragmenting process. A two-week acclimation period at baseline temperature (28°C) was chosen as per previous work with the same colony (Santos and Shaw n.d.).

Each tank was equipped with a Fluval E100 100W heater, or Eheim 300W heaters in high temperature tanks (to ensure stable temperature exposure), a Koralai Nano 420 circulation pump, and an individual AquaticLife light fixture. Each light fixture contained two fluorescent bulbs: one Giesemann 24W Actinic blue bulb, and one 24W Giesemann Powerchrome Tropic bulb. These two bulbs were selected because together they mimic natural light settings experienced by reef systems. Light fixtures were suspended on a horizontal rod above the tanks to ensure photosynthetically active radiation (PAR) was kept constant between the tanks (approximately 60-80nm). Lights were set on an 8-hour photo period regime from 11:00 to 17:00. Opaque black corrugated plastic sheets were placed between each tank to stop any bleeding of excess light between the tank glass. Tanks were kept at 28°C before the trials began to ensure healthy individuals.

During the entire experiment, tanks were maintained by conducting water changes and ensuring the tanks remained clean. Water was changed in the tanks when it became murky or a film gathered on the surface. Salinity and temperature were constantly measured for consistency, and tanks were topped up with reverse osmosis water when low (RiOs 100 reverse osmosis system (EMD Millipore, Billerica, Massachusetts, USA)).

3.1 Thermal Stress Trials

Over the course of nine weeks, from 27 January to 30 March, 2020, each of the four experimental tanks were brought to four different thermal maximums: 29, 30, 31, and 32°C. The control tank was kept at 28°C throughout the entire length of the experiment. The purpose of sequential thermal treatments was to distinctly monitor corals at different temperatures and compare them simultaneously. The experimental design also avoided subjecting all coral fragments to maximum fatal temperatures, resulting in death of all the samples. Each tank underwent a heat increase, thermal maximum, and a recovery phase (as seen in Table 1), these distinct phases were called baseline, thermal maximum and recovery respectively. By segmenting the corals into different thermal maximum trials, it allowed for a precise determination of the bleaching/death temperature of the corals, while comparison to other thermal levels was still possible. On the first day of each week, temperatures were changed in the tanks by 1°C, according to their specific thermal schedule. For example, tank 3 was brought up 1°C every week until reaching its thermal maximum at 30°C, then was brought down every week until reaching 28°C (baseline temperature).

3.2 Fluorescence Measurements and Sampling

During the heat treatment trials, sampling was conducted twice per week on the fifth and seventh day, wherein one fragment was randomly sampled from both the control and trial tanks. Samples were collected around the same time every day for consistency (approximately 13:00-14:00). Selected fragments were placed into an acclimation tank near the fluorescence microscopy workstation for 30 minutes minimum. The purpose of the tank was to reduce stress to the corals when moving the individuals, and for ease of access for imaging. After the acclimation period, coral fragments were collected in glass dishes and placed under a fluorescent

microscope for imaging. Images were taken of randomly selected tentacles of sampled corals. An Olympus SZ61 Zoom Stereo Microscope attached to an Olympus XM10 Monochrome fluorescence CCD camera, was used to take images of the corals (exposure time = 0.98ms, gain = 12.5dB, resolution =1376x1038, 2.5X magnification). Images were taken in a fluorescent spectrum with an X-Cite Series 120Q EXFO at an excitation of 630nm (as per Santos and Shaw). Blue violet was used because it allowed for clear imaging and distinction of the red fluorophotopigment produced by the dinoflagellate symbionts and the green protein produced by the coral tissue. Four different images of each tank's coral fragments were digitally collected on an Olympus cellSens Standard software. On ImageJ (National Institutes of Health, Bethesda, Maryland, USA), each of the four images were analyzed four separate times, and an average of all 16 measurements was taken to determine average fluorescence of each tank. This measurement process was repeated for each treatment tank. Fluorescence measurements were calculated by subtracting the background reading from the measured pixel intensity of the tentacle. Fluorescence intensity was calculated with a specific formula of "Integrated Pixel Density of Tentacle - (Background Reading x Area of Measurement)". After all measurements were taken, corals were returned to a larger marine tank and were not used again.

3.3 Colour-Health Index

Using a "Coral Watch" Coral Health Chart (The University of Queensland, Australia), sampled corals were assessed on an ordinal scale to represent differing coral tissue colour. On a scale of 1-6, 1 being completely colourless and bleached and 6 is the darkest and healthiest colour. Corals were ranked on this scale and assigned numbers as a proxy for individual health. The card was held to the proximal end of individual tentacles to measure colour.

3.4 Statistical Analysis

All statistical analyses were conducted using the statistical software SPSS 25 (IBM Inc., Armonk, New York, USA). To determine an overall trend of how fluorescence changes with increased temperature, data from the "Thermal Maximum" phase of each of the five thermal trial groups was compared via One-Way ANOVA. Post-hoc analysis of a Tukey-Kramer multiple comparisons was done on measurements that were found to be significantly different. To determine recovery ability for each of the four thermal treatment regimes, comparison was done between the baseline, thermal maximum, and recovery phases. Data points from the 28°C of the baseline, the thermal maximum, and the 28°C measurements of the recovery phase, were compared to for each thermal trial. For the control, the first, middle, and last fluorescent measurements were compared for consistency. To compare these three treatment phases, a One-way ANOVA was used followed by a post-hoc Tukey-Kramer multiple comparisons. By comparing the before, during and after heat treatment it could be determined if the corals were able to successfully recover. If no statistical difference was found between the baseline and recovery, but both were significantly different from the thermal maximum group, recovery would be considered to be achieved.

For tissue colour-health index, a linear regression was used to determine the relationship between change in temperature and the colour of coral tissue. No further analysis was done within treatment groups due to a small sample size (n=6).

4. Results

Between the four thermal trial groups and the control, there was a statistically significant difference in fluorescence values at the thermal maximum temperatures (One-Way ANOVA: $F_{4,155}=164.33$, $P<0.05$). Post-hoc Tukey-Kramer analysis showed that all thermal groups were

significantly different from the control ($P<0.05$ for all groups, Figure 1). However, 29°C and 30°C thermal groups were the only heat treatments where no statistical difference was found between treatment groups ($P=0.61$), all other groups were significantly different in pair-wise comparison ($P<0.05$ for all). Overall, there is a downward trend observed in fluorescence intensity as temperature treatments were increased (Figure 1).

Analysis within the five treatment groups successfully determined where recovery success was achieved. Within the control (28°C), there was not a significant difference between beginning, middle, and end of the trials (One-Way ANOVA: $F_{2,93}=1.88$, $P=0.16$) (Figure 2A). Within the 29°C thermal maximum trial, there was a significant difference between the treatment groups ($F_{2,93}=23.50$, $P=5.49\text{x}10^{-8}$). Post-hoc Tukey-Kramer analysis indicated that both thermal maximum and recovery measurements were approximately 20% less than the baseline fluorescence ($P=1.97\text{x}10^{-8}$, $P=3.00\text{x}10^{-6}$, respectively). However, there was no difference between then thermal maximum and the recovery phase fluorescence readings ($P=0.40$, Figure 2B). Within the 30°C group, there was a strong and significant difference between groups ($F_{2,93}=139.53$, $P=1.00\text{x}10^{-28}$). Post-hoc analysis indicated that the thermal maximum florescence was 25% less than the baseline, whereas the recovery was 45% less fluorescent than the baseline (all group pairwise comparisons $P<0.05$, figure 2C). For the 31°C group, significant a difference was found between groups again ($F=51.80$, $P=7.62\text{x}10^{-16}$). Both the baseline and recovery phases showed approximately 38% more fluorescence than the thermal maximum (Tukey-Kramer: $P=5.10\text{x}10^{-9}$, $P=5.10\text{x}10^{-9}$, respectively). Most importantly, the baseline and recovery phases of this thermal trial group were not statistically different from each other ($P=0.57$, Figure 2D). Within the 32°C group, there was a significant difference between groups ($F=705.21$, $P=6.31\text{x}10^{-57}$), wherein the thermal maximum fluorescence was approximately 70% less than the

baseline, but the recovery phase was only 50% less than baseline (all pairwise comparisons $P<0.05$, Figure 2E).

For colour-health indices, linear regression indicated a moderately strong negative relationship between colour rank and temperature (Pearson Correlation value= -0.62, $R^2=0.379$) as seen in Figure 3.

5. Discussion

The significant difference in fluorescence intensity between all five thermal maximum groups was a predictable outcome. As established in previous studies, corals will expel endosymbionts during periods of thermal stress, which is likely to be exacerbated with higher temperatures (Ralph et al. 2001). Loss of endosymbionts likely gave rise to the decrease in fluorescence intensity observed in the present study. However, an interesting phenomenon of note was a spike in fluorescence intensity noted at 30°C, followed by a sharp decline (Figure 1). This finding may be connected to previous work that also noted a similar rise-then-fall response of dinoflagellate density at similar temperature levels (Santos and Shaw n.d.). A possible explanation of this observation could be that a thermal compensation point is present at 30°C. However, because 29°C and 30°C groups were not significantly different, the thermal compensation process may start at the initial onset of thermal stress, but reach a pinnacle at 30°C, where after compensation is no longer possible. The proposed mechanisms of the compensation point are as follows: 1) dinoflagellate cells optimally operate in a 30°C temperature range, 2) this temperature could be a trigger point to produce more proteins as a last-resort protection mechanism against future thermal stress and 3) increased fluorescence is a by-product for an increase in photosynthetic activity. Support for the first point has been explored in previous studies wherein dinoflagellates of numerous species seem to thrive in relatively high

temperatures, and have been found to peak at 30°C (Granéli et al. 2011). Moreover, previous studies have also observed a rise-then-fall phenomenon in other dinoflagellate species as well, likely due to a peak in productivity around 30°C, then subsequent death at higher fatal temperatures (Hallegraeff et al. 1997). Estimates have suggested endosymbiotic dinoflagellates within coral species have a thermal maxima range between 30-34°C due to degradation of the photosystem and other proteins (Iglesias-Prieto et al. 1992, Warner et al. 1999).

Secondly, it has been found that coral species upregulate gene expression for proteins that are crucial in the response to oxidative stress, metabolism changes, and increased light levels (Edge et al. 2013). It is conceivable that the *Anthelia* in this study upregulated proteins with fluorescent properties in order to compensate for undergoing thermal stress. Increased expression of heat shock proteins is a common response to thermal stress in many coral species, and may be a tactic for adapting to higher temperature environments (Black et al. 1995, Sharp et al. 1997). The logic of thermal compensation through protein expression may also apply to the third proposed explanation, wherein there may also be upregulation of genes responsible for photosynthetic proteins. Increased ocean temperatures are commonly associated with an increase in ultraviolet (UV) radiation, which could lead to greater photosynthetic potential of dinoflagellates symbionts (Gleason and Wellington 1993). Such an increase in photosynthetic capabilities would likely lead to greater fluorescence and greater number of dinoflagellates. Further, coral hosts may provide extra support to endosymbionts, as they produce amino acid compounds that absorb broadband ultra violet light radiation, and enzymes with antioxidant activity to decrease photo-oxidation of symbionts (Lesser 2006). Although the rise-then-fall of fluorescence is neither in support nor refutation of the original hypothesis, it is an interesting

phenomenon that compliments the current field of coral-dinoflagellate research and provides strong assistance to the main findings in this study.

For the analysis of recovery capability within thermal groups, the 28°C group (control) having no difference between the three time periods shows that without thermal stress, corals will maintain a constant fluorescence (Figure 2A). However, notably even with a seemingly minor 1°C increase fluorescence values reduce drastically, which alludes to the responsiveness of the coral itself to thermal change. Within the 29°C thermal maximum tank, it is evident that the corals did not achieve successful recovery, as the recovery phase did not come back to baseline fluorescence (Figure 2B). This finding is in refutation of the original hypothesis because recovery was not achieved, but nonetheless, this should not suggest that a 1°C change is detrimental to the corals, as likely this finding was due to a short measurement period. A limitation to this trial was that the timeline was not long enough to capture the recovery of this thermal maximum. Likewise, in the 30°C trial it was evident that recovery was not accomplished (Figure 2C). This finding is also in refutation of the original hypothesis as recovery was not achieved. However, based the first analyses of the thermal maximum groups, the 30°C trial had the highest fluorescence (Figure 1). As such, 30°C is likely the trigger point for the thermal compensation process; wherein fluorescence increases in preparation for greater thermal stress to come, as seen in the subsequent thermal trial (31°C) where successful recovery was achieved. Thus, 31°C is the point of thermal stress where corals are not yet bleached and can successfully recover. This finding at 31°C is in support of the original hypotheses as the corals exhibited recovery capability. The compensation process may still have residual effects at 32°C, as this thermal trial also showed a slight recovery in florescence values but was not able to fully

recover, which supports the original hypothesis. However, if these trials were extended beyond 32°C, we would likely see even less of a recovery capability until the corals completely died.

Similar to the fluorescence data, colour health assessment showed the same peak at 30°C, which reiterates that at 30°C there is likely a compensation point. Overall, the negative trend observed in the colour-health index also supports the fluorescence findings as there was a decrease with increased thermal stress (refer to Figure 3 and 1, respectively). This finding suggests that fluorescence and tissue colour are closely linked. Because colour health indices have been used as a reliable proxy for prognosis of bleaching, perhaps this indicates that fluorescence is a novel but adequate method for determining coral health as well (Siebeck et al. 2006, Montano et al. 2010).

During the trials, some fragments displayed more resilience than others in the 32°C tank, as only a few were still alive during the peak of the thermal stress period. This is possibly due to genetic variation within the tank in the corals, symbionts or both, wherein the variation could have made some individuals better adapted, and thus better fit for a warmer environment (Rowan 2004). This is commonly seen in natural environments where some corals in a colony will not be affected as much as others (LaJeunesse et al. 2010). Because some tanks had substantially longer trial periods, this may have led to more time to cope with the thermal stress and adapt. As such, time may have been a confounding variable in the experimental design. Because some trials were shorter than others, perhaps equalizing all trial lengths would be beneficial. Improvement may also be made by replicating this study, as pseudo replication of cloned individuals impacts the applicability of the findings of this study and greatly reduces the sample size.

In terms of the larger field of coral research, understanding what the thermal maxima of specific coral species are, and that recovery is achievable in some cases, is very useful information. Knowing recovery capabilities could prove to be helpful in long-term modeling, government decision making in regard to climate action, or conservation efforts. Future extensions of this work could be aimed towards determining recovery capability of other coral species or determining how other stressors impact recovery such as heavy metals or ocean acidity.

6. Acknowledgements

I would like to acknowledge the MacEwan Lab Technicians who provided fundamental support to me throughout the process of my project, Kathy Davies as an honour's advisor, Ross Shaw for his mentorship, and to Zachary Vydra and my parents who have been my greatest encouragement.

7. Tables & Figures

Table 1: Experimental timeline of thermal trials in respective experimental tanks. Experimental phases outlined as Baseline (orange), Thermal Maximum (red) and Recovery (green) with associated temperatures and weeks.

Week	Control (Tank 1)	Tank 2	Tank 3	Tank 4	Tank 5
1	28°C	28°C	28°C	28°C	28°C
2	28°C	29°C	29°C	29°C	29°C
3	28°C	28°C	30°C	30°C	30°C
4	28°C	X	29°C	31°C	31°C
5	28°C	X	28°C	30°C	32°C
6	28°C	X	X	29°C	31°C
7	28°C	X	X	28°C	30°C
8	28°C	X	X	X	29°C
9	28°C	X	X	X	28°C

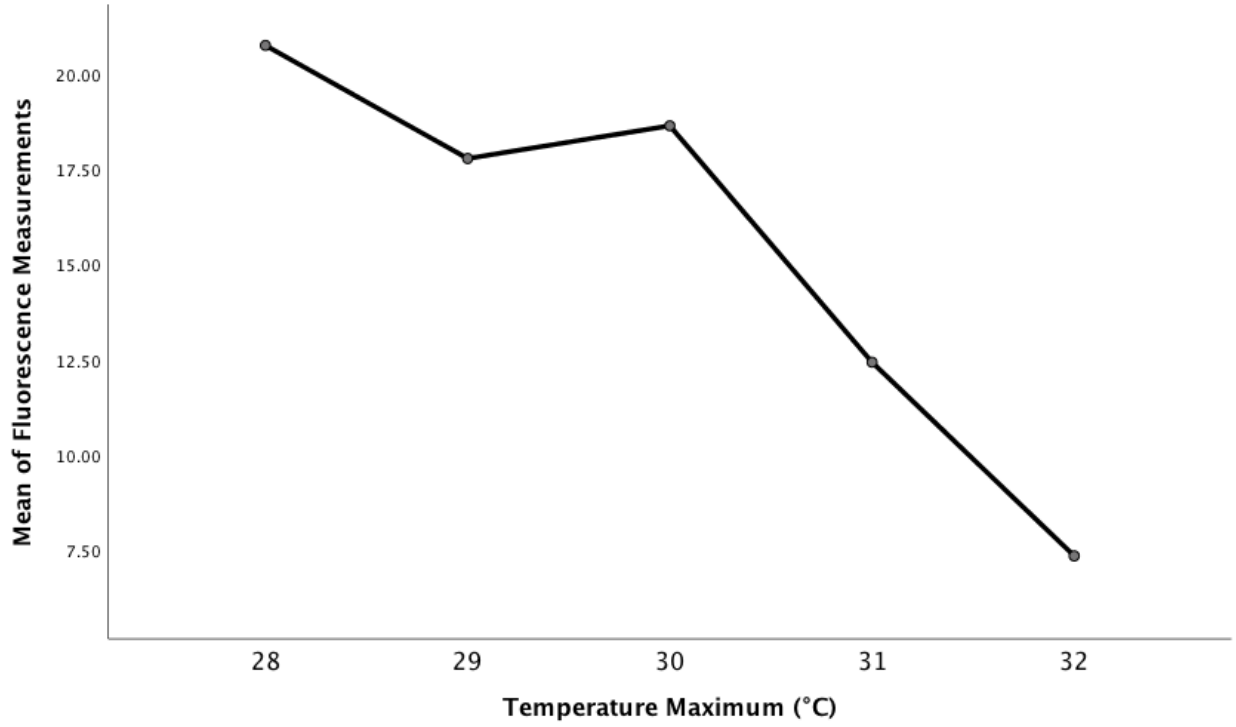

Figure 1: Line graph of mean fluorescence measurements of *Anthelia* sp. over the span of five thermal trials. Each point represents the average of the of fluorescent measurements taken during the thermal maximum phase of thermal trials (n=32). All five thermal groups shown to be statistically significant different from eachother (One-Way ANOVA: $F_{4,155}$=164.33, P<0.05), 29°C and 30°C groups not significantly different (P=0.61).

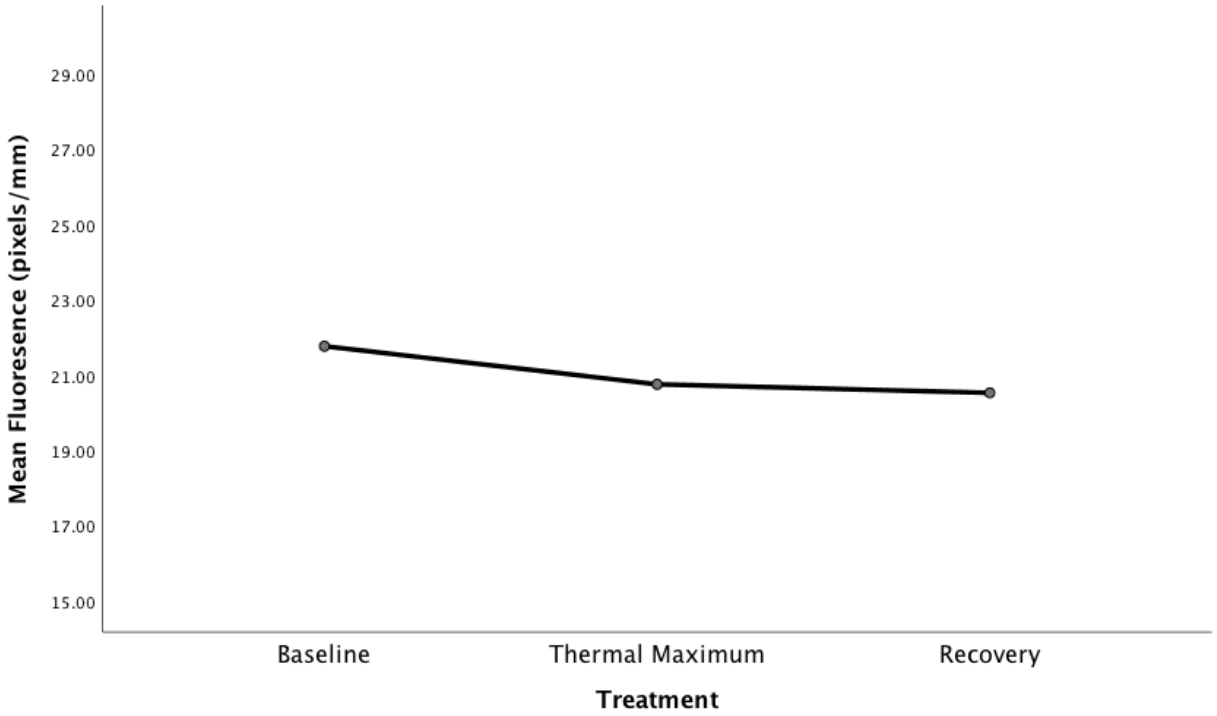

Figure 2A: Line graph of mean fluorescence readings of *Anthelia* sp. over the course of three experimental phases: Baseline (28°C), Thermal Maximum (28°C), and Recovery (28°C) of a thermal experimental trial (n=32). Significant difference not present between groups (P=0.16).

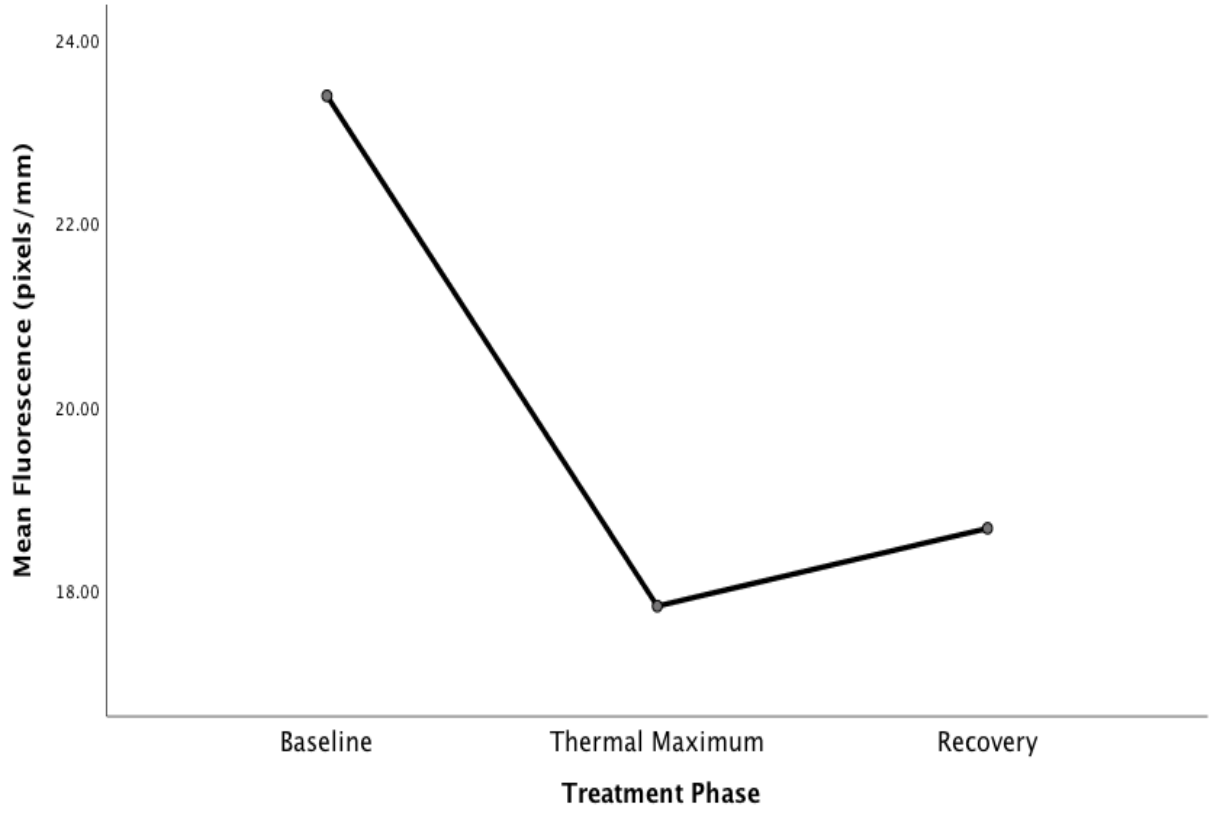

Figure 2B: Line graph of mean fluorescence readings of *Anthelia* sp. over the course of three experimental phases: Baseline (28°C), Thermal Maximum (29°C), and Recovery (28°C) of a thermal experimental trial (n=32). Baseline phase only group significantly different from all other groups ($P=1.97x10^{-8}$, $P=3.00x10^{-6}$ for thermal maximum and recovery groups, respectively).

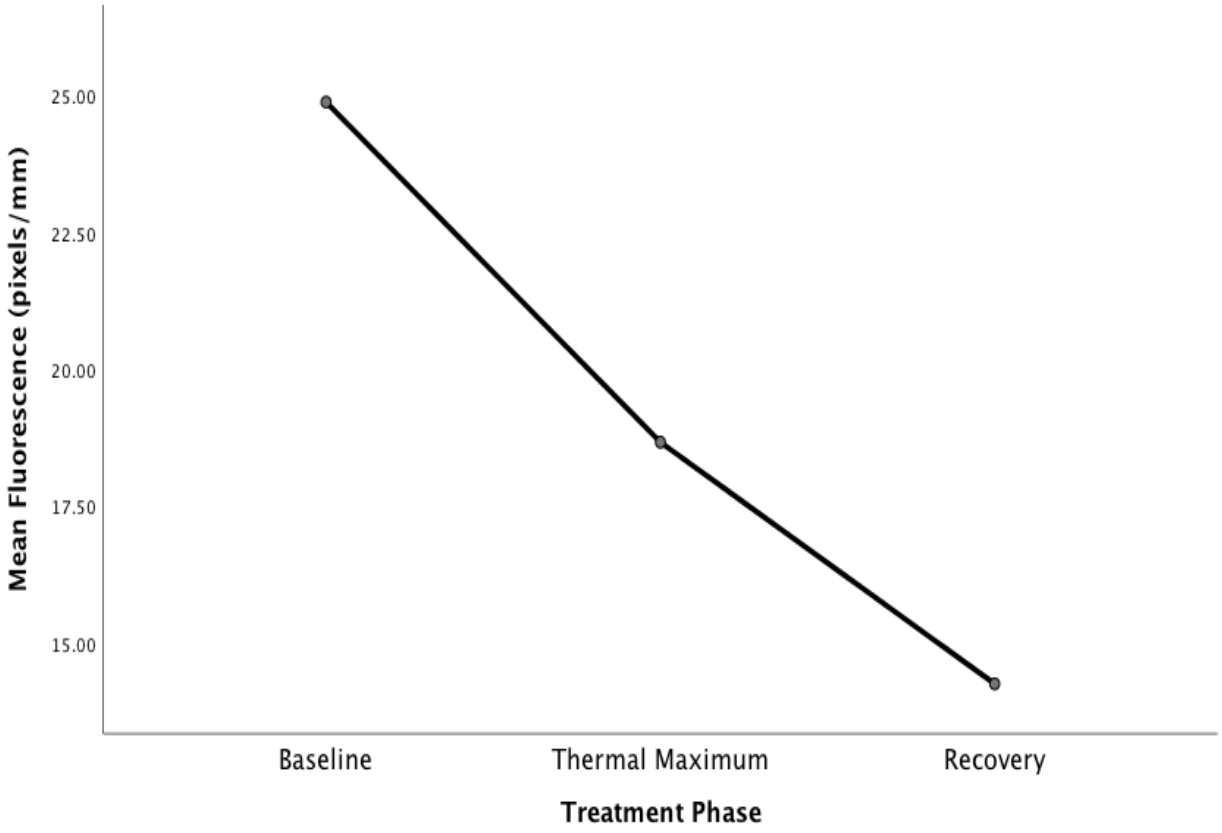

Figure 2C: Line graph of mean fluorescence readings of *Anthelia* sp. over the course of three experimental phases: Baseline (28°C), Thermal Maximum (30°C), and Recovery (28°C) of a thermal experimental trial (n=32). All groups significantly different from eachother ($P<0.05$ for all pairwise comparisons).

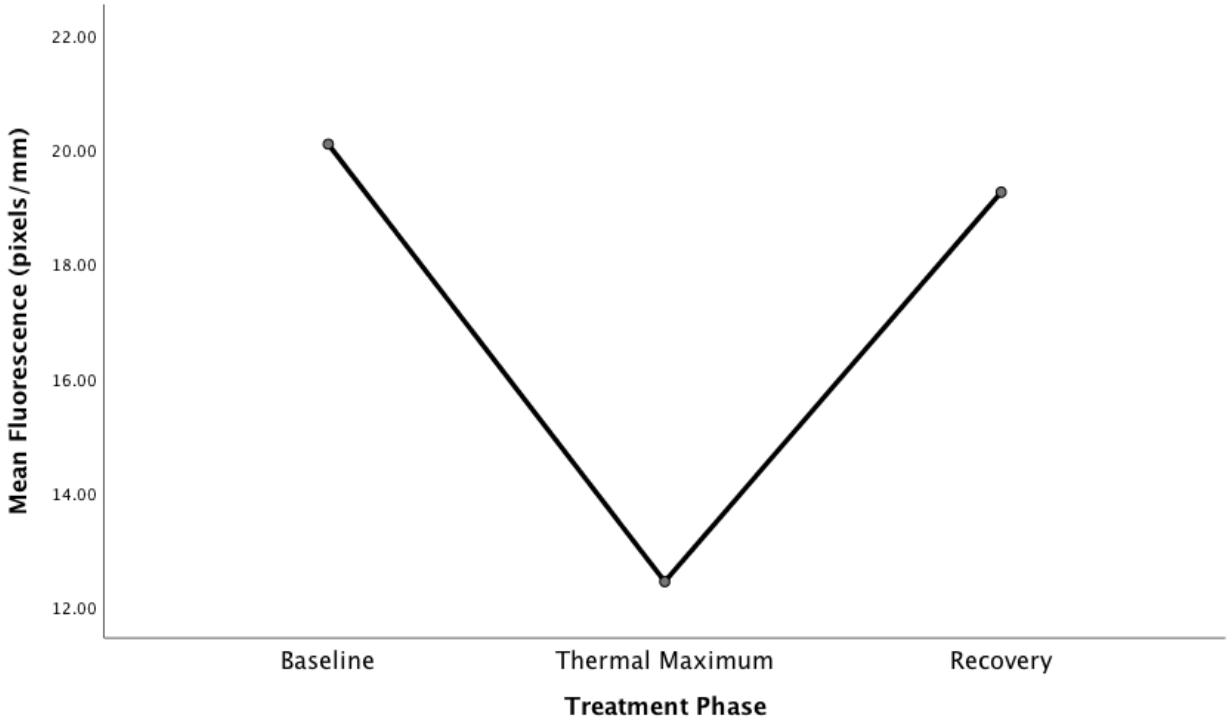

Figure 2D: Line graph of mean fluorescence readings of *Anthelia* sp. over the course of three experimental phases: Baseline (28°C), Thermal Maximum (31°C), and Recovery (28°C) of a thermal experimental trial (n=32). No significant difference found between baseline and recovery groups (P=0.57).

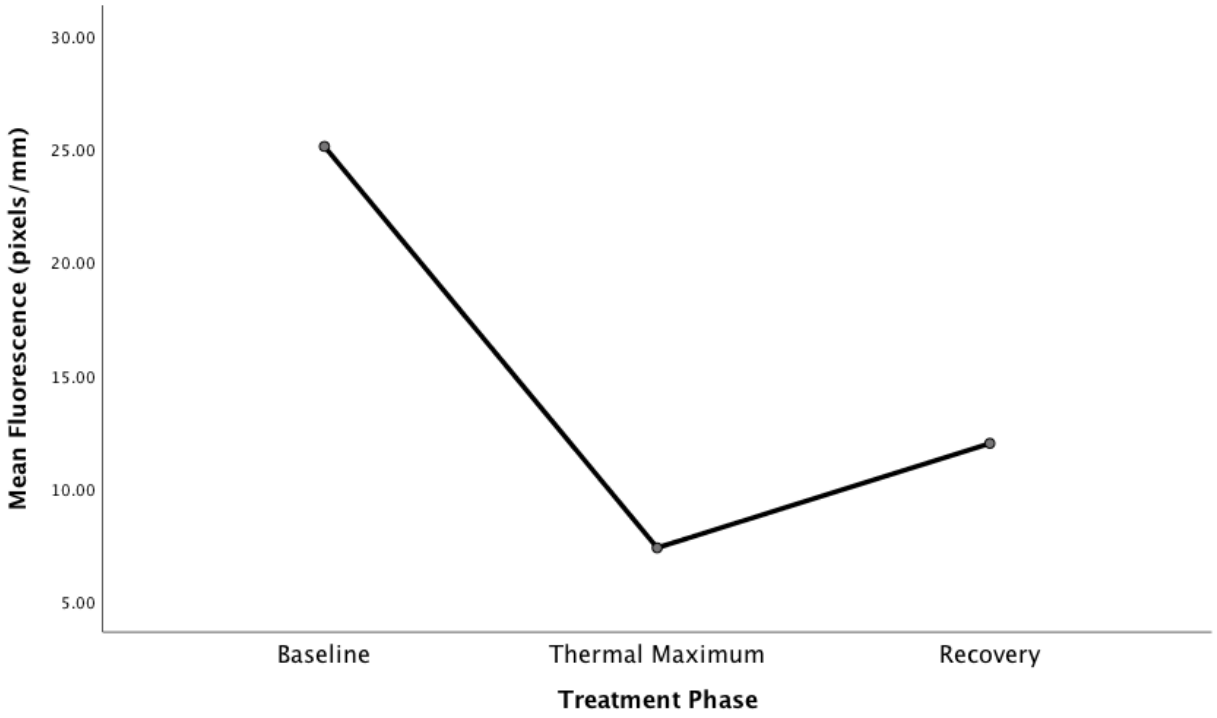

Figure 2E: Line graph of mean fluorescence readings of *Anthelia* sp. over the course of three experimental phases: Baseline (28°C), Thermal Maximum (32°C), and Recovery (28°C) of a thermal experimental trial (n=32). Statistically significant difference between all groups (P>0.05 for all pairwise comparisons).

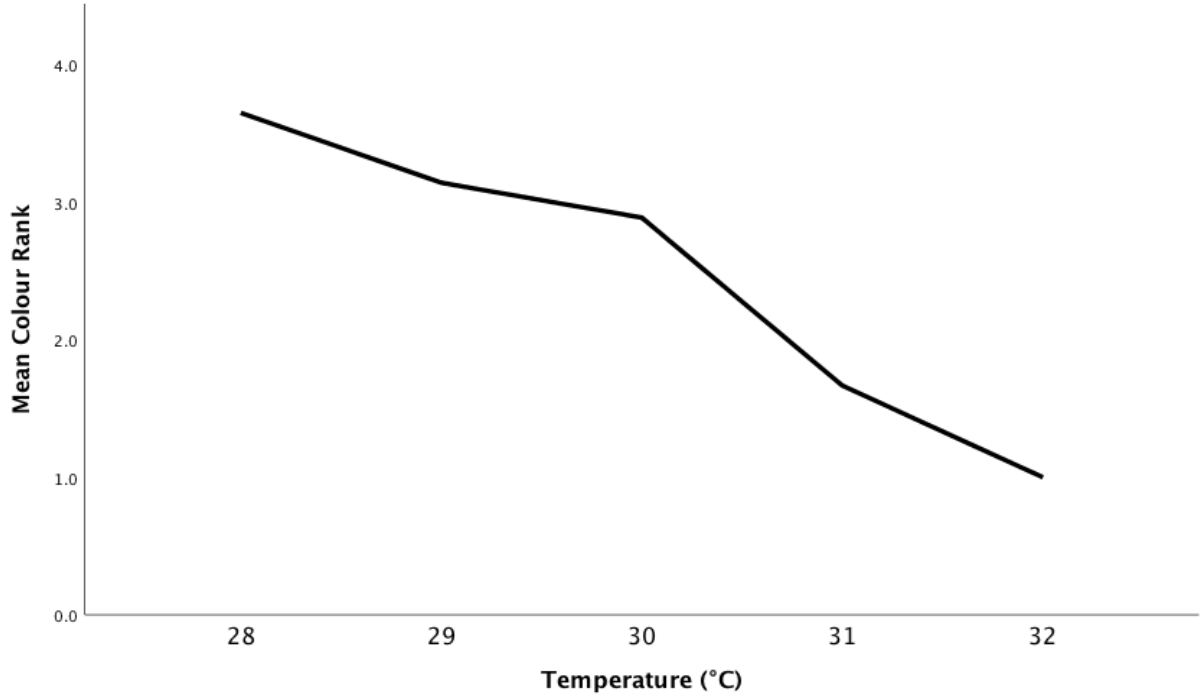

Figure 3: Line graph of linear regression analysis of mean colour-health index as a function of five temperature treatments of *Anthelia sp. (*n=64). (Pearson's correlation coefficient: r=-0.616, R^2=0.369).

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Appendix

Pilot Study

In order to make full certainty that the tanks and the individual lighting, heating, circulation systems etc. did not cause any extraneous variables to the study, while also allowing to ensure that each tank was a similar and reliable environment for sampling. Corals were placed in the experimental tanks over the span of six weeks from 25 November 2019 to 6 January 2020. Measurements were taken of the experimental tanks, while they remained at the same temperature of the baseline 28°C. Fluorescence measures were taken twice a week as per the experimental protocol, over the course of six weeks. There was found to be no statistically significant difference between the tanks (Randomized Block ANOVA: $F_{5,25}$=0.532, P=0.750).