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Differing temperature regimes have no impact on the heat stress response of shallow and upper-mesophotic populations of a temperate calcareous sponge

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Marine heatwaves (MHWs) are increasing in intensity and frequency globally owing to anthropogenic climate change. Temperate mesophotic ecosystems (TMEs; 30–150 m) are light-dependent habitats that generally experience cooler and more stable temperatures than adjacent shallow zones, potentially leading to intraspecific differences in thermal tolerance between shallow and mesophotic populations. Sponges, often the dominant benthic organisms in TMEs, show varied responses to thermal stress. We hypothesized that shallow-water sponges may be more resilient to heat stress than conspecifics from upper-mesophotic depths. To test this, we exposed *Rowella lancifera* sponges from one shallow and one upper-mesophotic population to a simulated MHW and measured respiration, buoyant weight, mortality and microbial community composition. Temperature records from the preceding year confirmed cooler, more stable conditions at upper-mesophotic depths. Sponges displayed strong stress responses under MHW exposure, resulting in high mortality, but responses did not differ between depths. Elevated respiration rates also coincided with microbial community shifts. Overall, our results indicate that a 1°C increase above previous MHW peaks could cause widespread mortality in *R. lancifera*.

1. Introduction

Marine heatwaves (MHWs) are increasing in frequency, duration and intensity globally as a result of anthropogenic climate change [1]. MHWs can persist from weeks to years, covering spatial scales from sub-regional to thousands of kilometres across multiple oceans, impacting at the scale of individual species up to whole ecosystems [2–4]. As thermal tolerance of marine ectotherms tends to roughly match their ambient temperature variability [5,6], the widest tolerance windows are generally found in temperate species, where seasonal variability in temperature is highest [7]. Wider temperature thresholds could mean temperate species are better equipped to deal with future warming events [8]. However, recent increases in heatwaves globally highlight the need for studies on the impacts of warming on temperate species, despite the view that they may be more resilient than their tropical counterparts [1,9].

Temperate mesophotic ecosystems (TMEs) occur in cool waters around the world and span depths at the limit of light availability for photosynthesis

[10,11]. Studies on mesophotic coral ecosystems typically define the mesophotic zone as ranging from approximately 30 to 150 m, based on factors such as irradiance levels and the absence of primary producers [12,13]. However, in temperate regions, seasonal fluctuations in local environmental conditions such as changes in temperature, sedimentation rates and rainfall can alter these boundaries [14,15]. Owing to the reduction of temperature with depth [16], previous studies have suggested that mesophotic ecosystems in the tropics, where water is stratified for longer [17], could escape some of the impacts of temperature increases and extreme temperature events [18,19].

Depth-generalist species may be less affected by MHWs than those inhabiting a narrow depth range, as they naturally persist under a wider range of temperatures [20]. However, as shallower populations of depth-generalists are likely to be living in warmer temperatures than those in mesophotic habitats, they may already be living closer to the upper limit of their temperature range [5,16,17]. There are cases where shallow-water populations of benthic, sessile organisms are affected by MHW events, while populations in TMEs remain unaffected [21–23]. These cases may result from mesophotic populations existing further below their thermal tolerance limits than shallow populations, or from lower extremes of MHWs at mesophotic depths [24]. In the tropics, it was previously suggested that unimpacted mesophotic populations may repopulate shallow waters after disturbance events such as MHWs [18]. However, the extent to which mesophotic populations are protected is often location- and species-specific [20,25]. Despite depth-generalists potentially being able to withstand a wide range of temperatures, intraspecific variation in thermal tolerance between populations caused by either genetic isolation or phenotypic plasticity may cause populations inhabiting shallow and mesophotic depths to be impacted differently [26,27].

Sponges are often one of the dominant benthic organisms in TMEs, where they can cover up to 70% of the benthos [28,29] and carry out a wide variety of important functional roles [30]. Thermal stress has been shown to impact sponge respiration rates [4], causing spicules to become deformed [31], affecting the sponge microbiome [32,33] and even causing bleaching and death [33,34]. However, owing to their long evolutionary history, some sponges may initially be more resilient to future environmental change than other organisms living within the same marine ecosystems [35,36]. For example, recent successive MHWs on TMEs in Australia and New Zealand caused mass bleaching and mortality of some sponge species, while many others were seemingly unaffected [33,34]. Owing to the wide variety of functional roles played by sponges in TMEs, it is important to understand their thermal limits and how likely it is that they will survive MHWs in the future.

In this study, we explored differences in thermal stress responses of the temperate calcareous sponge *Rowella lancifera* taken from one shallow (10 m) and one upper-mesophotic (30 m) population. We hypothesized that shallower habitats would be subject to warmer, more variable temperatures, compared with upper-mesophotic depths. As a result of this temperature difference, we expected shallow water sponges to exhibit lower levels of thermal stress response when compared with upper-mesophotic sponges. We also expected MHW conditions to disrupt the sponge microbiome, causing dysbiosis (an imbalance in the microbial community).

2. Methods

(a) Species and study area

Rowella lancifera [37] is a species of calcareous sponge that is common around temperate Australia and New Zealand, from the shallow subtidal to 450 m deep [28,38]. *R. lancifera* has a tubular form, with one apical osculum, elevated and surrounded by a membrane [39], and at our study site it reached a maximum apical length of roughly 10 cm (M. Broadribb, author observations, 2023). *R. lancifera* sponges were collected from Breaksea Sound in the Fiordland Marine Area (FMA; electronic supplementary material, figure S1). The FMA is made up of 14 fiords that are known for their highly diverse marine communities [40]. High annual rainfall (>6400 mm) and rapid runoff from steep topography create a tannin-rich low-salinity layer (LSL), driving sharp depth gradients in hydrographic and biogeochemical properties [41,42]. In some cases, the LSL can reduce levels of solar irradiation to 1–2% of surface levels within just 15 m [43], meaning the upper limit of the mesophotic zone begins at shallower depths in the FMA than in other systems. The steep, vertical walls typical of fiord systems [44] cause shallow and mesophotic communities to be separated spatially by depth alone, allowing analysis of depth-dependent responses without the influence of additional confounding spatially driven factors.

(b) Temperature data collection

Temperature loggers were deployed at 10, 30 and 50 m at one site in Breaksea Sound (electronic supplementary material, figure S1). These recorded temperatures every 10 min from their deployment on the 6th of January 2022 until the 5th of August 2023. For sea surface temperature records (used in the calculation of MHWs for the area) the National Oceanic and Atmospheric Administration (NOAA) 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) was utilized [45]. The OISST is a long-term climate record that incorporates data from satellite, ship and buoy observations into a global grid, interpolated to provide complete global maps of sea surface temperature (SST) [45,46]. As NOAA OISST data are at 1/4° resolution, grid points nearest the logger and sponge collection sites were used, noting that the SST may not precisely match local conditions.

(c) Sponge collection and maintenance

Owing to logistical challenges, sponges were collected from a second site in Breaksea Sound (45°34'06.6"S 166°46'00.5"E), which was 2.51 km away from the temperature logger site (45°34'23.3"S 166°47'53.9"E) (electronic supplementary material, figure S1). Sponges were collected using SCUBA from adjacent shallow (10 m) and upper-mesophotic (30 m) depths in one dive on the 21st of March 2023 ($n = 48$ for each depth). Dive knives were used to pry the sponges from the substrate, keeping the base intact to minimize damage. Sponges were then placed in ziplock bags while still underwater to ensure no contact with the air and transferred into thermally insulated bins. The bins contained seawater collected from the sampling site and were set up with aquarium aerators to ensure the water remained oxygenated, with temperature monitored and regulated to maintain 17–18°C, and water changed regularly throughout transportation. On the following day, sponges were transferred into large carrying containers and secured in a cooler for transportation back to Victoria University Coastal Ecology Lab (VUCEL) in Wellington, NZ.

At VUCEL, sponges were placed in 50 l tanks ($n = 16$ per tank) and attached to small ceramic tiles using aluminium wire, ensuring they were in an upright position (electronic supplementary material, figure S2). Tanks were set up to allow a flow of 10 μ m filtered seawater for the duration of the 10 day acclimation period and the 31 day experimental period (flow rate: 35 ± 4 l h⁻¹) based on the methods of Strano *et al.* [4]. Water temperature in the tanks during the acclimation period was set to 18°C for all tanks to match the temperature from which the sponges were collected.

(d) Experimental conditions

Sponges were exposed to two temperature regimes (control and MHW), with each replicated three times ($n = 6$ experimental tanks). Sponges were randomly selected and distributed between tanks ($n = 8$ from each depth per tank). Control tanks were maintained at 18°C for the duration of the experiment, with temperature in treatment tanks gradually increased to simulate MHW conditions (electronic supplementary material, figure S3 and table S1). Water temperature was controlled by an aquarium controller system (Apex Classic, Neptune Systems—California, USA), connected to aquarium heaters (100 W, Eheim—two in each tank) and chillers (HC-500 A, Hailea) (electronic supplementary material, figure S4). Water temperature was also checked using a YSI Pro30 probe every day to calibrate the Apex system.

Respiration rate and buoyant weight were taken on days zero and one (T0), before increasing the temperature in treatment tanks. Water temperature in the treatment tanks was increased by 0.3°C per day for six days post-acclimation, reaching 19.8°C to match the peak of the most recent MHW in Breaksea Sound. Physiological measurements were taken on days seven and eight (T1). Temperature was then raised by 0.3°C for five days, with a final increase of 0.2°C on the sixth day, peaking at 21.5°C on day 14, with measurements taken on days 15 and 16 (T2). Temperature was maintained at 21.5°C for six days with final measurements on days 23 and 24 (T3), followed by a recovery period with temperature reduced by 0.5°C per day down to 18°C (electronic supplementary material, figure S3 and table S1). As all but three MHW treatment sponges suffered mortality by the end of the recovery period, no further measurements were taken. Our simulated MHW peak was based on the MHW that occurred in early 2022 in Breaksea Sound, but with a peak of 1°C warmer. The previous heatwave peaked at 20.23°C, 4.6°C above the seasonal average, and cumulated in 288.64°C-days (degree heating days [47]). Our simulated MHW peaked at 21.5°C, 5.6°C above mid-summer seasonal average and, based on the MHW threshold for mid-summer seasonal average, cumulated in 93.4°C-days.

(e) Survival and health monitoring

Sponges were monitored daily for the duration of the experiment, including monitoring any changes in morphology. Sponges showing signs of necrosis and/or severe tissue degradation (electronic supplementary material, figure S5) were considered dead and removed from their treatment tanks during daily checks.

(f) Buoyant Weight

To determine changes in sponge buoyant weight (BW), all sponges were weighed at T0 (before the beginning of the experiment), T1 (at 19.8°C), T2 (at 20.3°C) and T3 (at 21.5°C). BW was measured with a digital scale (A&D FX-200i) following the methods of Osinga *et al.* [48], whereby the sponge would remain fully submerged during weighing. The relative change in sponge BW in response to temperature variation for each time point (Ti) was calculated using equation (2.1).

$$\text{BWChange} = [(\text{BWTi} - \text{BWT0}) / \text{BWT0}] \times 100. \quad (2.1)$$

(g) Respiration rate

Respiration rates of all control and treatment sponges were measured at T0 (before the beginning of the experiment), T1 (at 19.8°C), T2 (at 20.3°C) and T3 (at 21.5°C) (electronic supplementary material, figure S3). Measurement protocols were based on those of Strano *et al.* [4]. Sponges were randomly selected and placed in sealed 250 ml cylindrical glass respiration chambers with PreSens oxygen sensor spots (SP-PS3-NAU) attached to their inner surface. A submersible magnetic stirrer ensured continual water movement within chambers, which were placed in a water bath with temperatures corresponding to

environmental conditions (either control or MHW) for the duration of the respiration measurements. After 15 min of acclimation (a sufficient period to acclimatize sponges according to preliminary trials), oxygen concentration inside the chambers was measured every 15 min for 45 min, using a Fibox 4 oxygen probe. We defined 6 mg l⁻¹ as moderately hypoxic and therefore the threshold below which measurements would stop, based on water temperature and salinity [49]; however, this threshold was never reached. To record possible microbial respiration in seawater, one blank measurement per temperature per time point was included for every incubation. At the end of the experiment, the surviving sponges ($n = 50$) were dried for 72 h at 60°C and then combusted at 500°C for 5 h. A linear regression was then carried out to convert BW measurements to ash-free dry weight (AFDW) (electronic supplementary material, figure S6), before sponge respiration rate was standardized to sponge ash-free dry weight (mgO₂ gAFDW⁻¹ h⁻¹) [50].

(h) Microbial community analysis

Samples of sponges (0.5 cm³) were collected with a sterile scalpel and forceps from control ($n = 5$) and MHW conditions ($n = 5$) on day 20 (mid MHW peak), before being flash-frozen in liquid nitrogen and stored at -80°C. Microbial DNA was then extracted using the DNEasy PowerSoil Pro Kit following the manufacturer's instructions (Qiagen, Germany). Nanodrop and gel electrophoresis on 1% agarose were used to assess the concentration (5.3 – 90.55 ng µl⁻¹) and quality of the extracted DNA.

Amplicon library construction and sequencing were performed by the Ramaciotti Centre for Genomics at the University of New South Wales, Australia, on the MiSeq V2 platform (Illumina). To target the V4 region of the 16S rRNA gene, modified primers 515 f (5'-GTGYCAGCMGCCGCGGTAA-3') [51] and 806 r (5'-GGACTACNVGGGTWTCTAAT-3') [52], containing Illumina overhang adapter sequences, were used. The full PCR protocol for DNA amplification and 16S rRNA library preparation is provided in the supplementary information. Raw sequences were filtered, trimmed and primers removed using USEARCH [53] version 11.0.667 and TRIMMOMATIC [54] version 0.38, as previously described by Strano *et al.* [32]. Amplicon sequence variants (ASVs) were generated using UNOISE3 [55], and chimerae were removed with UCHIME2 [56] in reference mode. For taxonomic assignment of ASVs, the Bayesian Last Common Ancestor (BLCA) algorithm [57] was applied to compare sequences against the SILVA database v. 132 [47], generating a microbial species list for each sponge sampled under control and heatwave conditions.

(i) Statistical analyses

All statistical analyses were performed in R v. 3.1.3 [58]. For all multiple comparisons, p -values were corrected using the Benjamini–Hochberg (BH) procedure [59]. The corrected p -values were used to determine statistical significance at an alpha level of 0.05. Plots were created using 'ggplot2' [60].

OISST data were analysed using the heatwaveR package [61] to define MHW metrics in the three years prior to sponge collection. Several criteria are used for defining MHWs [1]. Here, we use the definition given by Hobday *et al.* [62], with MHW metrics calculated 'relative to a baseline period (climatology) and a particular time of the year from which the intensity, duration and spatial extent of the MHW could be defined'. Temperature logger data were averaged to give daily mean temperatures. A Levene's test was carried out to examine differences in temperature variance between depths, followed by Tukey's HSD tests for pairwise comparisons between depths [58]. As the Levene's test result was significant, a Welch's ANOVA was carried out to determine differences in mean temperatures between depths, followed by a Games–Howell test for pairwise comparisons between depths. Finally, a linear model was created, and contrasts in trends in temperature through time between depths were calculated using emtrends [63].

For both respiration rate and BW analyses, we used linear mixed-effects models with normally distributed errors and random intercepts [64]. In all mixed models, treatment and time were considered fixed effects, while experimental chamber and sponge specimen were considered random effects. For these models, fixed- and random-effect terms were tested using the functions *anova* and *ranova*, respectively, while *post hoc* pairwise comparisons were computed on estimated marginal means using emtrends [63]. Respiration rates and BWs were log ($x + 1$)-transformed to meet normality assumptions. The goodness of fit, normality and homoscedasticity of the errors were checked for all models by inspecting plots of the normalized residuals and the quantile–quantile plots. Time to event analysis for sponge survival was performed using the Kaplan–Meier method, and p -values were calculated using the log-rank test implemented in the survival R package [65].

Differences in microbial communities were visualized using n MDS (non-multidimensional scaling) plots based on Bray–Curtis dissimilarities. Differences in community structure were analysed using permutational multivariate analysis of variance (PERMANOVA) [66], with differences in dispersion of communities tested using permutational multivariate analysis of dispersion (PERMDISP) [67] based on Bray–Curtis dissimilarities. Models were run using 9999 unrestricted permutations. To identify significant changes in the abundance of specific microbial taxa, the ANCOM-BC2 package was used [68]. Alpha diversity indices of microbial communities (Richness, Shannon, and Simpson) were calculated on microbial sequences rarefied to the minimum sequencing depth using 100 iterations, through the *phyloseq_mult_raref_div* function [69]. Differences in diversity indices between treatments at each time point were then explored using linear models and estimated marginal means [58,63].

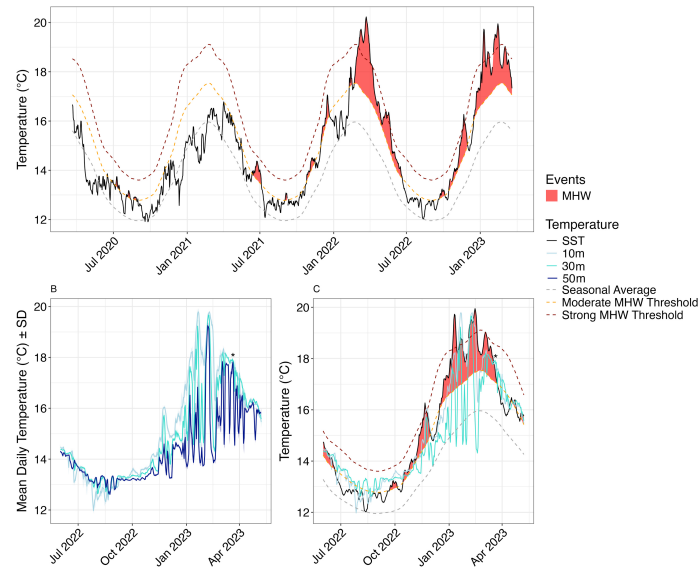


Figure 1. (A) Daily mean SST (black line) based on NOAA OISST data from the $1/4^\circ$ area closest to our sampling sites between May 2020 and March 2023. The dashed black line indicates the climatological mean, the orange dashed line represents the threshold for a moderate MHW and the red dashed line represents the threshold for a strong MHW (based on [61]). (B) Mean daily temperatures $\pm$ s.d. from temperature loggers at 10, 30 and 50 m from June 2022 to September 2023. (C) Mean daily temperatures from loggers at 10 and 30 m overlaid on data from graph B covering the same time period.

3. Results

(a) MHW analysis of SST

There were a total of 13 heatwaves that occurred in the $1/4^\circ$ area covered by the NOAA SST data closest to both our temperature logger and sponge collection sites over the 3 years prior to our sponge collection date. Of these, the highest-intensity MHW began in February 2022, lasting 120 days with a mean intensity of 2.41°C above the seasonal average and a maximum intensity of 4.63°C above the seasonal average, cumulating in $288.64^\circ\text{C-days}$ and peaking at 20.23°C . The second-highest intensity MHW began in December 2022, lasting 99 days with a mean intensity of 2.78°C above the seasonal average and a maximum intensity of 4.58°C above the seasonal average, cumulating in $275.68^\circ\text{C-days}$ and peaking at 19.73°C (figure 1A).

(b) Temperature logger data

At 10 m depth, mean daily temperature throughout the logging period was 15.23°C , with a variance of 4.38°C ; at 30 m, mean daily temperature throughout the logging period was 14.90°C , with a variance of 3.09°C ; and at 50 m mean daily temperature throughout the logging period was 14.36°C with a variance of 1.92°C (electronic supplementary material, table S2). Levene's tests showed that variance in temperature differed significantly between depths, and pairwise comparisons showed that variance in temperature differed significantly between all pairs of depths (electronic supplementary material, table S2). A Welch's ANOVA showed that mean temperature differed significantly between depths, and Games–Howell pairwise comparisons showed significant differences in mean temperature between all pairs of depths (electronic supplementary material, table S3). On average, the temperature at 30 m was 0.33°C cooler than 10 m, and at 50 m was 0.54°C cooler than 30 m (figure 1B, electronic supplementary material, table S3). Temperature showed a gradual positive trend through time at all depths, although temperature trends were found to differ significantly across all pairs of depths (electronic supplementary material, table S4). Although we were unable to detect MHWs exactly from logger data owing to the time frame not allowing calculation of a seasonal average, both 10 m and 30 m (depths from which sponges were collected) passed the MHW threshold based on SST during all MHW events during the logging period (figure 1C).

(c) Mortality rate

Within the treatment tanks, all but three sponges suffered mortality by the end of the experiment, with mortality beginning on day 20 (temperature 21.5°C during MHW peak) and continuing through to day 30 (temperature 18.5°C during recovery phase). One sponge kept under control conditions suffered mortality on day 30, but all other control sponges survived the experiment. Kaplan–Meier analyses showed the probability of sponge survival significantly decreased with time under thermal stress conditions (figure 2A), but there was no difference in survival probability between shallow and upper-mesophotic sponges within this treatment (figure 2B, electronic supplementary material, table S5).

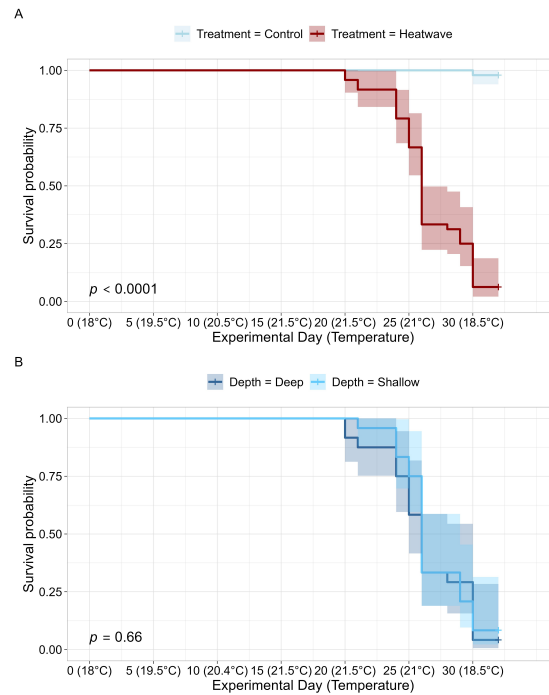


Figure 2. Kaplan–Meier curves for probability of sponge survival at different time points (A) under control and thermal stress conditions and (B) under thermal stress conditions in sponges collected from upper-mesophotic (30 m, deep) and shallow (10 m, shallow) sites.

(d) Respiration rate

Time, depth and the interaction between treatment and time, and time and depth, all significantly affected sponge respiration rate (electronic supplementary material, table 14). The respiration rate significantly decreased through time for both upper-mesophotic and shallow control sponges, while no significant change in respiration rate was detected for sponges from either depth subjected to MHW conditions (figure 3A, electronic supplementary material, table S6). Pairwise comparisons showed changes in respiration rate between control and treatment sponges differed significantly over the course of the experiment in both upper-mesophotic and shallow sponges, with respiration rate relatively higher in MHW-treated sponges (electronic supplementary material, table S6). Pairwise comparisons between depths showed a trend towards differences in respiration rate through time between shallow and upper mesophotic sponges under thermal stress; however, this trend was not statistically significant (BH-corrected $p = 0.056$; electronic supplementary material, figure S7 and table S7).

(e) Buoyant weight

Depth, and the interaction between treatment and time, and treatment and depth, significantly affected relative sponge buoyant weight change, despite time and treatment alone having no effect (electronic supplementary material, table S8). Relative change in sponge buoyant weight significantly increased through time for control sponges and significantly decreased through time in heatwave-treated sponges, yet when looking at shallow and upper-mesophotic depths individually, relative change in buoyant weight became non-significant under both control and thermal stress conditions (figure 3B, electronic supplementary material, table S9). Pairwise comparisons showed that changes in relative buoyant weight of shallow sponges under MHW conditions were lower than those of control sponges but found no significant difference in relative buoyant weight between upper-mesophotic and shallow sponges under either treatment (electronic supplementary material, figure S8 and table S10).

(f) Changes in morphology

Sponges exhibited changes in morphology under simulated MHW conditions. Sponges exposed to MHW temperatures exhibited signs of tissue regression, resulting in the exposure of external spicules and increased rugosity of the sponge surface, as well as signs of necrosis (electronic supplementary material, figure S4A–D). Many sponges under control conditions ($n = 28$) grew basally and were able to attach to the tiles to which they were secured, to the point where they were no longer being supported by the wires that were initially used to hold them into place (electronic supplementary material, figure S4E–G).

(g) Microbial community analysis

A total of 1 628 574 raw reads, 481 389 filtered reads (with mean read number $48\,138 \pm 13\,246$ (s.d.) per sample—electronic supplementary material, figure S9) and 125 ASVs were obtained for the V4–16S rRNA gene from five control and five MHW-treated sponges. PERMANOVA results showed that microbial communities at the ASV level differed significantly between sponges under control and thermal stress conditions during the peak of the simulated MHW on day 20 (figure 4A, electronic supplementary

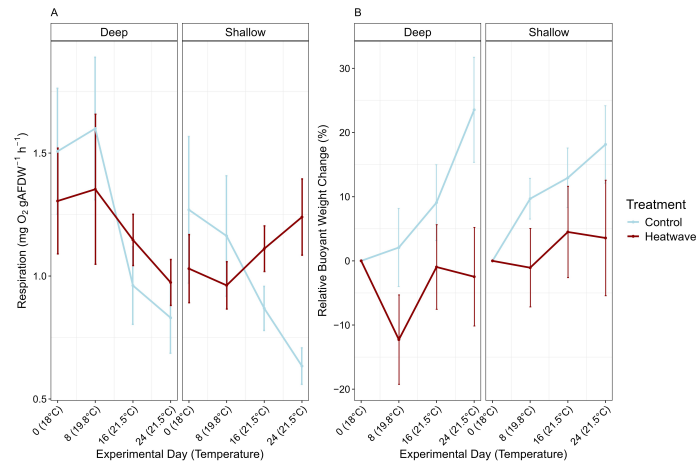


Figure 3. Mean ($\pm$ s.e.) (A) respiration rate, and (B) buoyant weight change, of upper-mesophotic (collected from 30 m) and shallow (collected from 10 m) sponges under control and thermal stress conditions at different temperatures throughout the experimental period.

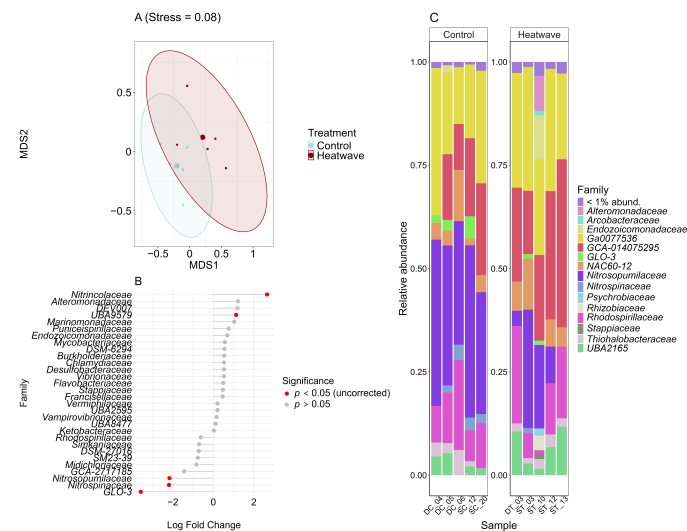


Figure 4. (A) nMDS plot showing differences in microbial community structure of sponges under control and thermal stress conditions. Large circles represent group centroids, small circles individual samples and ellipses 95% confidence around the centroid. (B) Results from ANCOM-BC2 analysis showing log fold changes in the 30 microbial families that showed the highest $\log(x+1)$ -fold changes in abundance between control and thermal stress conditions. Positive changes indicate higher abundances under MHW conditions. Significant results from uncorrected p -values are shown, although no p -values were significant after p -value correction. (C) Relative abundances of microbial families in each sponge sampled, taken from control and thermal stress conditions.

material, table S11). Results from the PERMDISP analysis showed no significant difference in distances of communities of individual samples from group centroids between control and MHW-treated sponges (electronic supplementary material, figure S10 and table S11). Relative abundances of the most common microbial families within the samples are presented in figure 4B. Although PERMANOVA results indicated overall differences in community structure, ANCOM-BC2 analysis revealed trends of higher relative abundance of *Nitrospiraceae* and *UBA9579* and lower relative abundance of *Nitrospumilaceae*, *Nitrospinaceae* and *GLO-3* in MHW-treated sponges compared with controls. However, these trends were not statistically significant after BH correction (Figure 4B, electronic supplementary material, table S12). Results from diversity index analysis found no significant differences in microbial ASV richness, Simpson's diversity or Shannon diversity between control and MHW-treated sponges (electronic supplementary material, figure S11 and table S13).

4. Discussion

This study aimed to assess whether stress responses would differ between sponges taken from shallow and upper-mesophotic populations of the calcareous sponge *Rowella lancifera*. We found that temperature varied significantly between depths just 20 m apart, and that shallow, upper-mesophotic and mesophotic depths all experienced temperatures above the minimum threshold for MHW events based on long-term climatic means calculated from SST data. Stress responses of sponges from both shallow and upper-mesophotic depths were significantly impacted by MHW conditions, with 95% of sponges suffering mortality before the end of the experimental period. Despite differences in temperatures between depths, we found no differences in stress responses between shallow and upper-mesophotic sponges. Our results show that a MHW with a peak 1°C warmer than those that had impacted the Fiordland Marine Area (FMA) in previous years would probably result in mass mortality of *R. lancifera*.

(a) Thermal stress responses

We found that all but three sponges under MHW conditions suffered mortality at some point between day 20 (the middle of our simulated MHW peak at 21.5°C) and the end of the recovery period at day 31. Owing to mortality only occurring after the 21.5°C temperature peak was reached, we hypothesize that the upper thermal limit of both the shallow and upper-mesophotic sponge populations lies somewhere in between 20.3°C (the peak of the early 2022 MHW) and the peak of our simulated MHW at 21.5°C. Being able to accurately predict the upper thermal limits of marine organisms is a crucial part of successfully projecting their persistence and potential distribution under future warming and extreme temperature events [70]. The results from this study can be used to anticipate the impacts of future MHWs affecting the FMA; however, accurate determination of critical thermal maxima requires a dynamic acute method, incorporating slope of the thermal ramp and exposure time [71].

Rather than temperature alone, the observed results may reflect cumulative effects of the successive MHWs that affected the FMA in the months and years prior to sampling (see figure 1A), pushing *R. lancifera* closer to its upper thermal limits. Fox *et al.* [72] proposed that successive MHWs may cause species to increase their heat tolerance. However, if there is not enough recovery time between MHW events, the accumulation of stress may negate any beneficial gains [73]. Recovery time between MHW events may vary across species and habitats, with shallow sponges probably experiencing faster temperature fluctuations than deep sponges [13], potentially influencing their ability to recover. Although direct data on recovery times for *R. lancifera* are lacking, studies on other temperate sponge species suggest that partial recovery from thermal stress can occur within weeks [4], and recovery from bleaching within a year [33]. As we sampled sponges immediately following an MHW (figure 1C), it may be the case that the accumulated stress from previous *in situ* MHWs, rather than the temperature of our simulated MHW itself, caused sponges to suffer mortality [74]. As well as mortality, there were differences in respiration rate through time between control and MHW-treated sponges, a response that often occurs in sponges under thermal stress conditions [4,75]. Increases in sponge respiration rates are often an indicator of acute environmental stress, as metabolic processes are altered in an attempt to adapt to the non-optimal conditions [75].

BW change through time differed significantly between sponges under control and heatwave conditions. Interestingly, the BW of control sponges increased throughout the experiment, which, coupled with the decrease in respiration rates, suggests that the sponges were acclimating to the experimental conditions. We found that many control sponges began to affix themselves to their tiles over time (electronic supplementary material, figure S5). Some calcareous sponges have a notable rigid, calcite basal skeleton [76], which is present in *R. lancifera* (M Broadribb, author observations, 2023) and could explain the increase in BW seen as sponges began to affix to tiles. Sponges under MHW conditions showed no change in BW through time, none of them attached basally and, when compared with control sponges, there was a relative decrease in BW. The tissue regression seen in MHW-treated sponges through the increase in visible spicules, as well as the degrading tissue visible in sponges in the treatment tanks, are also indicative of temperature stress [4,77].

There was a significant difference in microbial community structure between control and MHW-treated sponges, providing evidence that a shift was occurring as a result of temperature stress. Although the microbiomes of *Calcarea* have been less explored than those of *Demospongia*, global analyses have shown that these two classes share similar taxonomic groups, which probably carry out similar functions within sponges [78]. We observed trends towards differences in the abundances of five microbial families between control and heatwave-treated sponges, although these trends did not remain statistically significant after adjusting *p*-values for multiple testing. Because ANCOM-BC2 is conservative, particularly with small sample sizes such as ours [68,79], we highlight microbial families that showed potential trends of differing abundance between treatments, although these did not reach statistical significance. Both *Nitrosopumilaceae* and *Nitrospinaceae*, which are aerobic ammonia-oxidizing archaea and nitrite-oxidizing bacteria, respectively [80,81], had lower abundances in MHW-treated sponges when compared with controls. Conversely, *Nitrocolaceae*, whose members have the genetic capacity for anaerobic respiration through denitrification [82], were more abundant under MHW conditions when compared with controls. Respiration rates were relatively higher in sponges under MHW conditions, and this could limit oxygen availability and hence growth for the aerobic *Nitrosopumilaceae* and *Nitrospinaceae*, but allow for anaerobic bacteria, such as *Nitrocolaceae*, to thrive. A consequence of this would be a reduction in the removal of toxic ammonia from the sponges' bodies, which could negatively affect their growth [83]. The family UBA9579 also increased in abundance under MHW conditions. This family belongs to the class *Vampirovibrionia*, which unlike closely related cyanobacteria, lack the genes for photosynthesis and are known to thrive in anaerobic conditions [84]. Some members of this class have also been classified as predatory bacteria of microalgae [85] and their increased abundance may therefore have the potential to cause further dysbiosis in the microbiome, although there are no records of microalgae being found within *R. lancifera* and no records currently of UBA9579 displaying predatory behaviour. As we sampled our sponges before the end of the simulated MHW, it is possible that we were witnessing the beginning of a shift in microbial community structure that continued throughout the duration of the MHW. It is well documented that heat stress can cause shifts in sponge microbial communities [32,33]; however, the secondary effects of these shifts are less well studied.

(b) Prior MHWs

The MHWs that occurred in Breaksea sound in the FMA during the year prior to our sampling date were extreme, with the highest peak being 4.6°C higher than the seasonal average in the area. Unfortunately, as there are no prior studies estimating the age of *R. lancifera*, we are unable to say for certain whether they survived two consecutive heatwaves. However, as sponges were collected immediately following the MHW, which occurred in early 2023, the sponges used in our experiment must have

survived at least one extreme MHW. As many *R. lancifera* sponges survived these extreme temperatures, it may be that other temperate sponges are also resilient to temperature increases and MHWs. As *R. lancifera* is a depth-generalist, we propose that their survival through previous MHW events indicates that species inhabiting broader temperature ranges may be better equipped to deal with MHW events [21]. However, our results highlight that more intense future MHWs [1] may cause mass mortality of *R. lancifera* sponges within the FMA.

(c) Temperature *in situ*

Temperature logger data showed that even between depths just 20 m apart, mean temperature and temperature variance can differ considerably. Shallow (10 m), upper-mesophotic (30 m) and mesophotic (50 m) depths all differed significantly in mean temperature, temperature variance and temporal trends in temperature. Although the differences in temperature may seem small (less than 1°C in mean and less than 1.2°C in variance between 10 and 30 m), our results suggest that even a 1°C increase can push sponges above their upper thermal limits. Consequently, one might expect thermal tolerances to differ between populations inhabiting shallow versus upper-mesophotic depths [5]. However, on steep reef walls such as those in the FMA, shallow and mesophotic habitats are separated primarily by depth, resulting in short distances between populations that could facilitate strong genetic connectivity [86]. If populations are genetically well connected, any differences in adaptation to temperature stress would need to occur within a generation [87]. This is plausible in our study populations, given the generally long life spans of sponges and the repeated MHWs documented here [33,88].

Differences in thermal tolerance between shallow and mesophotic populations in other studies have been attributed to a combination of environmental variability, local acclimatization and selective pressures: shallow habitats experience larger and faster fluctuations in temperature, which can select for increased plasticity or enhanced stress-response mechanisms [89–91]. By contrast, mesophotic environments are typically more thermally stable, which may reduce the selective pressure for high thermal tolerance [18]. Nevertheless, not all species exhibit depth-dependent differences, as local adaptation, gene flow and life-history traits can modulate responses to thermal stress [92]. The absence of strong differences in response between depths in our study could reflect a combination of high genetic connectivity, similar exposure to repeated heatwaves, and the capacity of both populations to tolerate short-term thermal stress.

(d) Depth-dependent differences

As we were only able to sample sponges from one shallow and one upper-mesophotic population, we cannot make broad speculations that populations of *R. lancifera* from these depths in other locations, or under different temperature regimes, would respond similarly. Although we found no difference in stress responses between shallow and upper-mesophotic sponge populations, other studies investigating differential responses to MHWs of populations inhabiting shallow and mesophotic depths have found contrasting results. For example, Torrents *et al.* [90] found that shallow populations responded better to heat stress, Godefroid *et al.* [91] found that mesophotic populations responded better to heat stress, and Gould *et al.* [92] found that responses did not differ. A recent study by Rajman-Nagar *et al.* [89] on tropical sponges found no difference in respiration rate between sponges taken from shallow and mesophotic depths and subjected to MHW conditions, with no signs of necrosis or mortality in any of their study sponges, indicating overall resilience to thermal stress in both shallow and mesophotic populations.

(e) Ecological consequences

In our study, over 95% of sponges exposed to MHW conditions suffered mortality, showing that an MHW with a peak just 1°C higher than those occurring in the FMA over recent years could potentially wipe out both shallow and upper-mesophotic populations of *R. lancifera* at our study sites. If mortality were to occur at this scale for multiple sponge species, it could lead to knock-on effects on ecosystem services in the area. As sponges cover up to 70% of the benthos in shallow and mesophotic temperate rocky reefs [11,28], the loss of habitat complexity alone would impact other species in the area [30,93]. As other sponge species were detrimentally affected by previous MHWs in the FMA [33], it is probable that *R. lancifera* would not be the only species to undergo a mass mortality event if we were to see an MHW that matched the peak of our experimental simulation.

5. Conclusions

Our findings show that although shallow and upper-mesophotic populations of *R. lancifera* in the FMA seem adapted to higher-than-average temperatures, they are only a 1°C increase in MHW intensity away from a potential mass mortality event. This threshold of just 1 or 2°C seems to be similar to other marine organisms, with populations already inhabiting the upper end of their thermal niche [5,94,95]. As MHWs are already becoming more frequent and intense globally [1], an event like this could probably occur in the near future. There is a chance that deeper populations of sponges may survive, as we noted that temperatures are less extreme at depth, but this may not be the case for long as MHWs increase in intensity around New Zealand [96]. Owing to their wide variety of functional roles, sponge mass mortality events could lead to ecosystem-wide impacts; it is therefore important to understand the upper thermal limits of ecologically important organisms to predict the outcome of future MHW events.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All data, raw sequences, and analysis scripts supporting this study are available in the Dryad Digital Repository [97]. Supplementary methods, tables, and figures are provided in the electronic supplementary material [98].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. M.B.: conceptualization, data curation, formal analysis, methodology, writing—original draft, writing—review and editing; A.R.: funding acquisition, supervision, writing—review and editing; T.T.: formal analysis, writing—review and editing; V.M.: formal analysis, writing—review and editing; F.S.: formal analysis, writing—review and editing; J.B.: conceptualization, funding acquisition, methodology, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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