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REPORT

# On the use of rapid acute heat tolerance assays to resolve ecologically relevant differences among corals

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**Abstract** Rapid acute heat stress assays are increasingly used to assess reef coral heat tolerance and identify resilient corals for research and restoration. However, concerns remain about (1) how representative they are of natural bleaching events, and (2) how reproducible they are in resolving differences in heat tolerance among corals. To address these gaps, we (1) compared rapid assays with an 8-day 'classic' bleaching experiment on *Acropora pulchra* genets (n = 20), and (2) tested and retested rapid assay responses of *Acropora cervicornis* (n = 85 genets) up to a year apart. In both species, rapid assays revealed a ~ 2.5 °C range in heat tolerance thresholds ($F_v/F_m$ ED50s) among genets, and these thresholds were strongly predictive of individual bleaching responses in the classic bleaching experiment (R = 0.74). Retesting genets with rapid assays showed ED50 differences < 0.99 °C in 90% of cases (median = 0.47 °C), and controlling for environmental effects reduced these to < 0.54 °C (median = 0.23 °C). Even so, large differences (> 0.93 °C) were needed between two individual genets to reliably retest in the same rank order, though smaller margins were reproducible for groups (e.g., 0.29 °C for groups of 10). These results demonstrate that rapid assays may not resolve fine-scale individual heat tolerance differences, but they can easily identify top- and bottom-performing individuals, and differential tolerance between groups of corals. These findings provide critical validation and quantitative guidelines for the use of rapid assays, which may transform coral heat tolerance research and its applications to reef restoration.

**Keywords** Coral bleaching automated stress system (CBASS) · Symbiodiniaceae · Chlorophyll fluorometry · Mo'orea · Florida Keys · Restoration

## Introduction

Coral bleaching associated with marine heatwaves is driving mass mortality of reef-building corals around the world (Hughes et al. 2017), with devastating consequences for coral reef ecosystems and their services, worth trillions of dollars annually (Costanza et al. 2014). Ocean warming due to climate change is increasing the frequency and intensity of marine heatwaves (Oliver et al. 2021), with annual severe bleaching conditions expected on most of the world's reefs this century even under optimistic climate scenarios (van Hooidonk et al. 2016). Consequently, heat tolerance is the key trait that will determine persistence of reef corals and ecosystems, and heritable variation in heat tolerance will determine their capacity to adapt in warming oceans (Dixon et al. 2015; Howells et al. 2022). Furthermore, efforts to accelerate adaptive responses (National Academies of Sciences and Medicine, 2019; van Oppen et al. 2015) and maximize climate resilience in restored populations (Morikawa and Palumbi 2019; van

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Oppen et al. 2017) depend on identifying heat-tolerant individuals by quantifying and comparing heat tolerance for large numbers of corals. Rapid and reliable assays for coral heat tolerance phenotyping are, therefore, critical to advancing scientific research, conservation interventions, and restoration programs focused on climate resilience.

To fill this need for rapid high throughput testing, acute heat stress assays have recently emerged as a promising tool for rapid determination of coral heat tolerance thresholds (Cornwell et al. 2021; Voolstra et al. 2020), with methods coalescing into a standardized experimental framework termed the Coral Bleaching Automated Stress System (CBASS; (Evensen et al. 2023)). These portable, fieldable, assays can be applied more rapidly and to many more corals than 'classic' experimental bleaching studies, which often require dedicated laboratories for long-term environmental control and weeks to months to complete (Grottoli et al. 2021). The high throughput and increased standardization of acute heat stress assays has advanced comparative analyses of heat tolerance thresholds among coral species (Evensen et al. 2022), sites (Voolstra et al. 2021), populations (Marzonie et al. 2023), and individuals (Cornwell et al. 2021), and has made possible large-scale field-based determinations of heat tolerance thresholds for hundreds of colonies over hundreds of kilometers in short timespans (Cunning et al. 2021; Marzonie et al. 2023). Such censuses may help to identify heat-tolerant individuals and lay the groundwork for further advances in research (e.g., genomic studies of heat tolerance) and restoration (e.g., managed selection and breeding of heat-tolerant corals).

However, the utility of rapid heat tolerance threshold determinations in achieving these goals hinges on how representative they are of more environmentally realistic heat stress exposures. CBASS assays expose corals to higher temperatures for shorter durations than they would typically experience in a natural heatwave, and aim to identify the $F_v/F_m$ ED50 (the temperature required to elicit a 50% reduction in maximum photochemical yield of photosystem II ($F_v/F_m$)) as a standardized metric of heat tolerance; (Evensen et al. 2021). While there is a long history of the use of $F_v/F_m$ as an indicator of stress to the symbionts, whether corals' ED50s from CBASS constitute a strong proxy for performance during natural bleaching events remains uncertain, though some studies indicate that responses may be similar (Evensen et al. 2021; Morikawa and Palumbi 2019; Voolstra et al. 2020). However, only two studies have directly compared colony-level differences in heat tolerance from CBASS and 'classic' bleaching experiments performed on the same coral genets, with mixed results (Klepac et al. 2023; Voolstra et al. 2020). Additional evidence is therefore needed to evaluate how well acute heat tolerance threshold determinations represent in situ heat tolerance of individual corals.

Another key to the utility of rapid assays is their reproducibility and comparability, especially when characterizing individual genotype-level variation in heat tolerance. In practice, due to constraints on biomass and time, individual genotypes may only be tested in one CBASS experiment, producing a single $F_v/F_m$ ED50 value with unknown variance or repeatability. While replicate assays on the same genets performed simultaneously may be highly reproducible (Dörr et al. 2023), it is not yet certain whether heat tolerance thresholds are reproducible across space and time. Indeed, environmental factors may drive acclimatization resulting in changes in heat tolerance (Cunning et al. 2021; Scheufen et al. 2017), making it difficult to assign differences measured at different times and places to genotypic or environmental effects. Therefore, to enable large-scale efforts to identify heat-tolerant corals, it is critical to evaluate the reproducibility of rapid heat tolerance measurements for individual corals, and how they may vary over space and time.

Here we address these knowledge gaps surrounding rapid assays of coral heat tolerance: first, by testing their ecological relevance with a paired 'classic' (8-day, moderate duration (Grottoli et al. 2021)) bleaching experiment on twenty genotypes of *Acropora pulchra* in Mo'orea, French Polynesia; and second, by testing their reproducibility by repeating CBASS tests on eighty-five genotypes of *Acropora cervicornis* in Florida, USA, up to one year apart. We show that rapid assays resolve individual differences in heat tolerance that are (1) highly comparable to those observed in a classic bleaching experiment, and (2) broadly reproducible, especially when differences are large and environmental variation is controlled. Based on these findings, we discuss recommendations and best practices for using rapid heat stress assays like CBASS to determine and compare heat tolerance thresholds of corals, and guide their application in research and restoration.

## Materials and methods

### CBASS vs. classic bleaching experiment

To compare coral heat tolerance threshold measurements from CBASS assays to those derived from a classic bleaching experiment, we conducted simultaneous experiments on 20 colonies of *Acropora pulchra*. On December 3rd, 2022, 20 colonies of *A. pulchra* were collected from a back-reef lagoon in Mo'orea, French Polynesia (− 17.488528, − 149.886532) between 1–2 m depth. Snorkelers collected 16 branch tips (~ 5 cm each) from each of the 20 colonies, which were separated by at least 8 m to decrease the chance of collecting clones. Fragments were collected in plastic bags and returned within 3 h to a water table at the Richard

P. Gump South Pacific Research Station where they were held overnight in running seawater at ambient temperature (28 °C). Eight fragments of each genet were randomly allocated to the CBASS experiment, and the other eight fragments to the classic bleaching experiment.

The CBASS experiment was run the day following collection in a set of eight ~ 18-L tanks (50 cm × 50 cm with 7 cm of seawater). Each tank received light at ~ 500 µmol photons $m^{-2}$ $s^{-1}$ from LEDs (Phlizon 165 W; measured with an Apogee underwater cosine PAR meter MQ-210), and incoming seawater from Cook's Bay at ~ 2 mL $s^{-1}$ (turnover = ~ 2.5 h), with powerheads for water circulation (SUNSUN JVP 530 GPH). Coral fragments were secured horizontally with surgical tubing to eggcrate racks, which were rotated hourly to eliminate tank position effects. One fragment of each genet was placed into each tank at 11:30, with all tanks starting at 28.5 °C. At 12:00, temperatures in seven of the tanks were ramped up over 3 h to target temperatures of 31, 33, 34, 35, 36, 37, and 38 °C, respectively, and held for 3 h, and then ramped back down over one hour to 28.5 °C (Fig. 1a). Heating profiles were achieved by a custom Arduino controller (Evensen et al. 2023) and aquarium heaters (Finnex TH-300W). The tanks were not equipped with chillers, so the ramp down was achieved by increasing the inflow of ambient seawater to 10–20 mL $s^{-1}$ in all tanks, balanced by the continued activation of the heaters in each tank as needed. Lights were turned off at 18:00 for the ramp down, and $F_v/F_m$ measurements were taken starting at 19:00 (once temperatures had returned to 28.5 °C) using an Imaging PAM fluorometer (saturating pulse intensity = 1, saturating pulse width = 120 ms, gain = 1). From the fluorescence image, two measurements of $F_v/F_m$ were extracted for each coral fragment.

The classic bleaching experiment began on December 4th 2022, the day following collection in a set of four ~ 88-L tanks (50 cm × 50 cm with 30 cm of seawater) with incoming seawater at ~ 20 mL $s^{-1}$ (turnover = ~ 1.2 h). LED lights (AquaIllumination Prime 16HD) were set to come on at 05:15, ramp up over one hour to 500 µmol photons $m^{-2}$ $s^{-1}$ for 11 h, then ramp down over one hour and turn off at 18:15. Two branches of each genet, glued (IC-Gel Insta Cure Cyanoacrylate Gel Coral Glue) upright on ceramic plugs, were placed into each of the four tanks at 16:15, with all tanks at ambient temperature (~ 27–28 °C). After a ~ 1.5-day acclimation period, two of the tanks were heated to 31 °C, while the other two tanks were maintained at ambient temperature (Fig. 1b). The heated tanks were kept at 31 °C for 2 days, then 32 °C for 2 days, then 33 °C for 3 days (Fig. 1b), and coral fragment positions within each tank were rearranged randomly each day. Maximum photochemical efficiency was measured for all corals nightly throughout the experiment, one hour after the lights went off, using an Imaging PAM fluorometer, as described above.

Heat tolerance thresholds from the CBASS experiment were calculated for each colony by fitting a dose–response curve for $F_v/F_m$ as a function of maximum tank temperature (3-parameter log-logistic fit using the *drc* package (Ritz et al. 2015) in R (R Core Team 2020)), as in (Cunning et al. 2021; Evensen et al. 2021). Outlier data points identified by high Cook's distance (> 0.25) were removed. The ED50

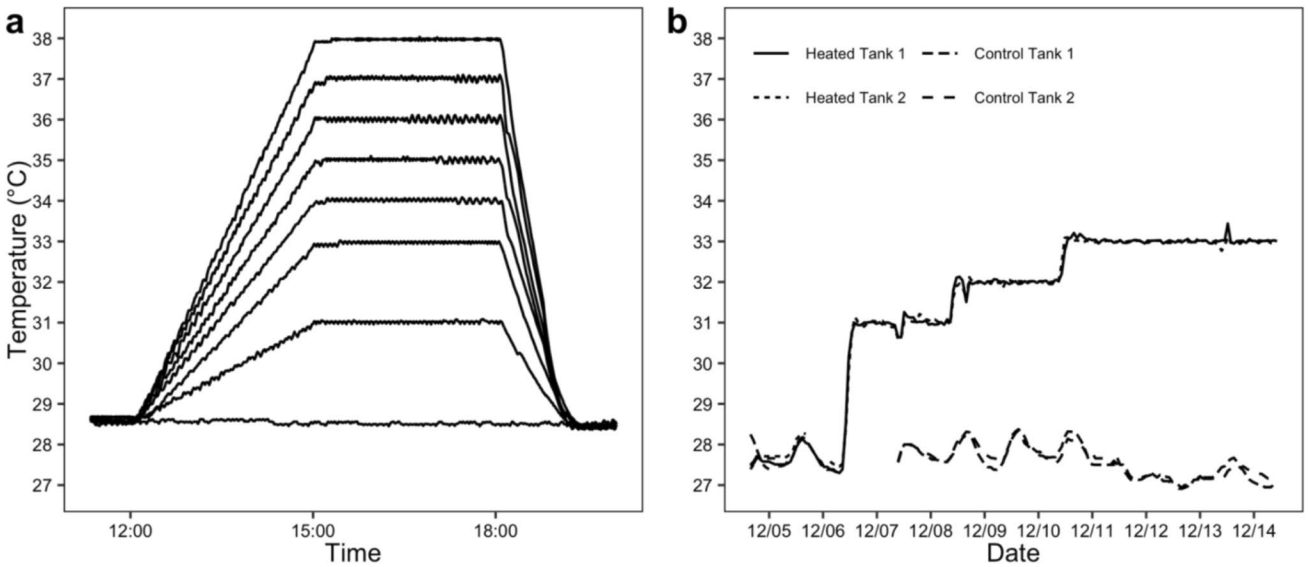


**Fig. 1** Temperature profiles measured in the CBASS experiment (**a**) and the classic bleaching experiment (**b**) on *Acropora pulchra*. In (**a**), the eight different lines correspond to the eight experimental tanks where temperatures were regulated by heaters and Arduino controllers. In (**b**), the two heated tanks were regulated in the same way, while the control tanks had ambient water temperature. The gap in temperature data from the control tanks was due to sensor malfunction

parameter (effective dose of heat stress required to reduce $F_v/F_m$ by 50%) was extracted for each colony as its heat tolerance threshold metric. In the classic experiment, $F_v/F_m$ measurements taken at the end of the experiment were analyzed with a linear mixed effects model (*lme4* package (Bates et al. 2015)) with treatment (control vs. heated) and colony as crossed fixed factors, and tank and fragment as random factors. From this model, the difference in $F_v/F_m$ between control and heated treatments ($\Delta F_v/F_m$) was calculated for each colony (*emmeans* package (Lenth et al. 2020)) as its heat tolerance performance metric. Heat tolerance performance metrics from the CBASS (ED50) and classic ($\Delta F_v/F_m$) bleaching experiments were compared across colonies with Pearson and Spearman correlation coefficients, and a Passing-Bablok regression for methods comparison studies (Passing and Bablok 1983).

### Reproducibility of CBASS assays

We performed a series of experiments to test reproducibility of CBASS ED50 over time in the same *A. cervicornis* genets. An initial test was conducted with 22 genets of *A. cervicornis* from the Mote Marine Laboratory coral nursery (Florida, USA) that were assayed in June 2020 and then again in October 2020, with these methods and results described in (Cunning et al. 2021). In September 2021, we conducted a larger test of reproducibility across four Florida coral nurseries (University of Miami (UM), Coral Restoration Foundation (CRF), Reef Renewal (RR), and Mote Marine Laboratory (MML)). Aboard the R/V *Coral Reef II*, we retested the heat tolerance of 67 *A. cervicornis* genets from these nursery stocks that were previously tested by CBASS in October 2020 (Cunning et al. 2021), approximately one year prior. At each nursery, SCUBA divers collected 8 branch tips (~5 cm in length) from each *A. cervicornis* genet using bone cutters. Corals were collected from the UM nursery on September 5th (n = 28 genets), held in the ship's livewells overnight in running seawater at ambient temperature, and assayed with CBASS on September 6th. Corals were collected from RR (n = 19 genets) and CRF (n = 10 genets) on September 7th and held in the livewells overnight. On September 8th, corals were collected from MML (n = 10 genets), and assayed together with the corals from RR and CRF.

CBASS assays were carried out on the R/V *Coral Reef II* as described in (Cunning et al. 2021). The experimental setup consisted of eight 16-L tanks (Coleman 24 Can Party Stacker coolers) with incoming seawater at a rate of ~2 mL $s^{-1}$ (turnover = ~2.2 h) and a submersible powerhead (SUNSUN JVP 530 GPH) to circulate water. Light was provided at 550 µmol photons $m^{-2}$ $s^{-1}$ in the center of each tank by LED lights (Phlizon 165W). Temperature was maintained by custom Arduino controllers connected to aquarium heaters (Finnex TH-300W) and chillers (Nova Tec IceProbe) in each tank. One fragment of each genet was allocated to each of the 8 tanks, and secured horizontally with surgical tubing to eggcrate racks, which were rotated hourly to eliminate tank position effects. Beginning at 13:40, seven tanks ramped up from 30 °C to seven different target temperatures (32, 33, 34, 35, 36, 37, 38 °C) over 3 h, then held those target temperatures for 3 h, while one tank remained at 30 °C. At 19:40, coinciding with local sunset, lights were turned off, and temperatures ramped back down to 30 °C over one hour. Maximum photochemical efficiency ($F_v/F_m$) was measured for each coral fragment using an Imaging PAM fluorometer, as described above.

ED50 parameters were estimated from dose–response curves fitted for each coral as described above (removing outlier points with Cook's distance > 0.25) and combined with those measured for the same coral genets from the same nurseries in June and October 2020 (Cunning et al. 2021). In total, 85 genets were tested, 81 of these twice, and 4 genets three times. All ED50 values (n = 174) were analyzed by a linear model with genotype and nursery:date as predictors, then filtered to remove 6 genotypes with Cook's distance > 0.035. ANOVA was used to quantify the significance and variance (proportion of sum of squares) explained by genotype and nursery:date. Adjusted ED50 values were calculated by adding residuals around (nursery:date)-specific means to the grand mean ED50, in order to remove variance due to nursery and date. Test–retest differences for each genet were then computed using both unadjusted and adjusted ED50 values and visualized as empirical cumulative distributions. To test whether differences in heat tolerance between coral individuals or groups of corals were reproducible, the mean difference between two groups of n corals was computed for 10,000 random permutations of each group size (n = 1, 3, and 10) with the October 2020 test data (using both adjusted and unadjusted ED50s). These differences (absolute values) were then used to predict whether the same two corals (or groups) were resolved in the same rank order by a second test (June 2020 or September 2021), using a generalized linear model with binomial errors.

## Results

### CBASS vs. classic bleaching experiment

The CBASS experiment on 20 colonies of *A. pulchra* revealed variable heat tolerance thresholds among colonies, with $F_v/F_m$ ED50 ranging from 34.3 to 36.6 °C (Figs. 2a, S1). The classic bleaching experiment also revealed variable heat tolerance among colonies, with declines in $F_v/F_m$ at the end of the experiment (heated—control) ranging from −0.09 to −0.49 (Figs. 2b, S2). These metrics of heat

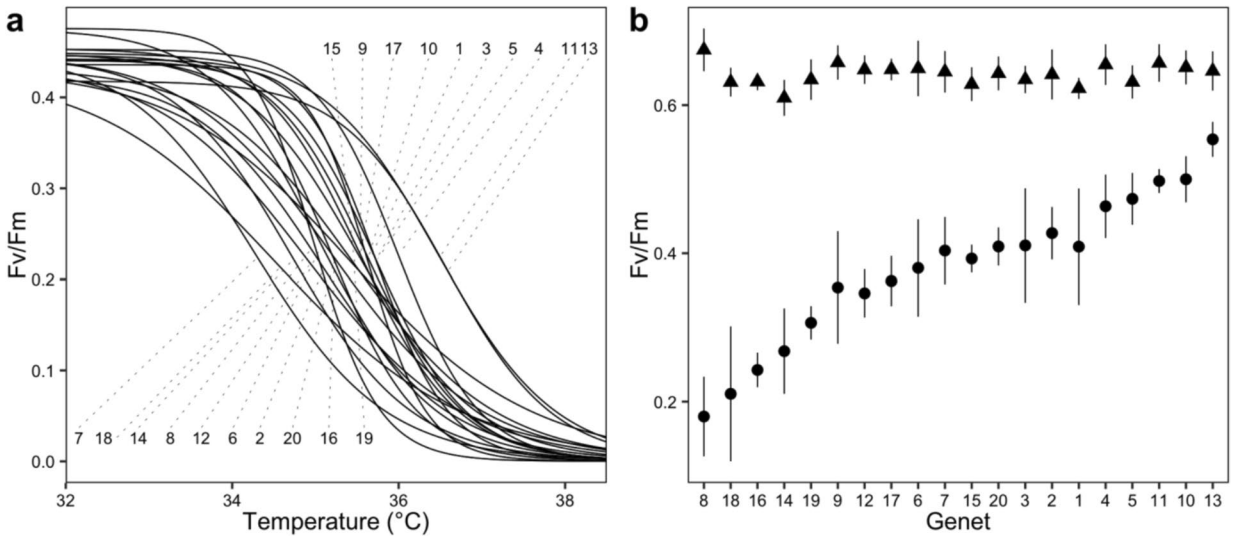


**Fig. 2** Thermal tolerance of each genet as measured by the CBASS experiment (**a**) and the classic bleaching experiment (**b**). The CBASS results (**a**) show the fitted dose–response curves for each genet, with the labels indicating genet identification numbers in order from lowest ED50 (genet 7) to highest (genet 13). The classic bleaching experiment results (**b**) show the mean $F_v/F_m$ measurements at the end of the experiment for each genet in the control treatment (triangles) and the heated treatment (circles), with error bars indicating standard deviation

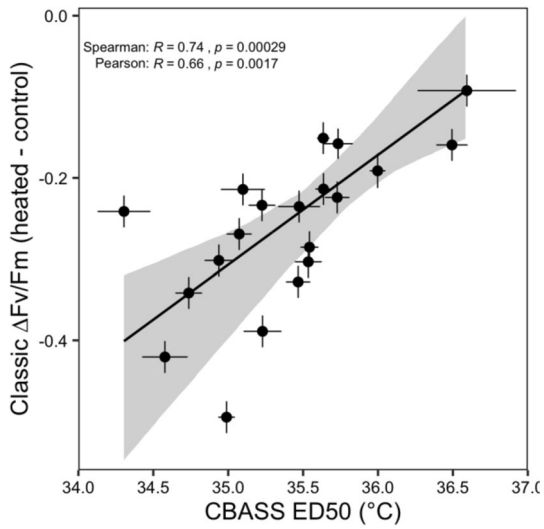


**Fig. 3** Comparison of CBASS and classic thermal tolerance measurements. Points represent individual genets, with error bars indicating the standard error of the ED50 parameter estimate (on the x-axis) and the standard error of the difference between heated and control treatments (on the y-axis). The line represents a Passing-Bablok regression (slope = −0.138) and shading is a 90% confidence interval

tolerance from the two different experiments were highly correlated across colonies (Fig. 3; Spearman's R = 0.74; Pearson's R = 0.66). Ordinary least squares regression found that a genet's CBASS ED50 value was strongly predictive of its response ($\Delta F_v/F_m$) in the classic bleaching experiment (Fig. 3; $p = 0.0017$).

The relationship between CBASS ED50s and $\Delta F_v/F_m$ in the classic experiment was not detected during the first 6 days of heat stress, when $\Delta F_v/F_m$ was smaller and similar across colonies. A correlation emerged on day 7 and became even stronger on the eighth and final day of the experiment (Fig. S3).

## Reproducibility of CBASS assays

Analysis of heat tolerance thresholds for individual corals that were tested and retested by CBASS up to a year apart (Fig. 4a) revealed that ED50 values depended significantly on coral genet ($p < 0.001$), and on the location and date of testing (nursery:date; $p < 0.001$). Coral genet explained 51.4% of the overall variation in ED50, while nursery and date explained 33.5%. Variation due to nursery and date (i.e., due to spatial and temporal environmental differences) was controlled for by adding residuals from nursery:date-specific means to the grand mean, producing adjusted ED50 values (Fig. 4b).

Test–retest differences for the same coral genet (at the same location, but up to a year apart), using unadjusted ED50 values, were < 0.47 °C in 50% of cases, and < 0.99 °C in 90% of cases. With adjusted ED50 values, test–retest differences were < 0.23 °C in 50% of cases, and < 0.54 °C in 90% of cases (Fig. 4c). On average, test–retest differences were reduced by 46% with adjusted ED50 values, relative to unadjusted values.

To test the reliability of comparing heat tolerance thresholds between genets from a single CBASS test, we computed

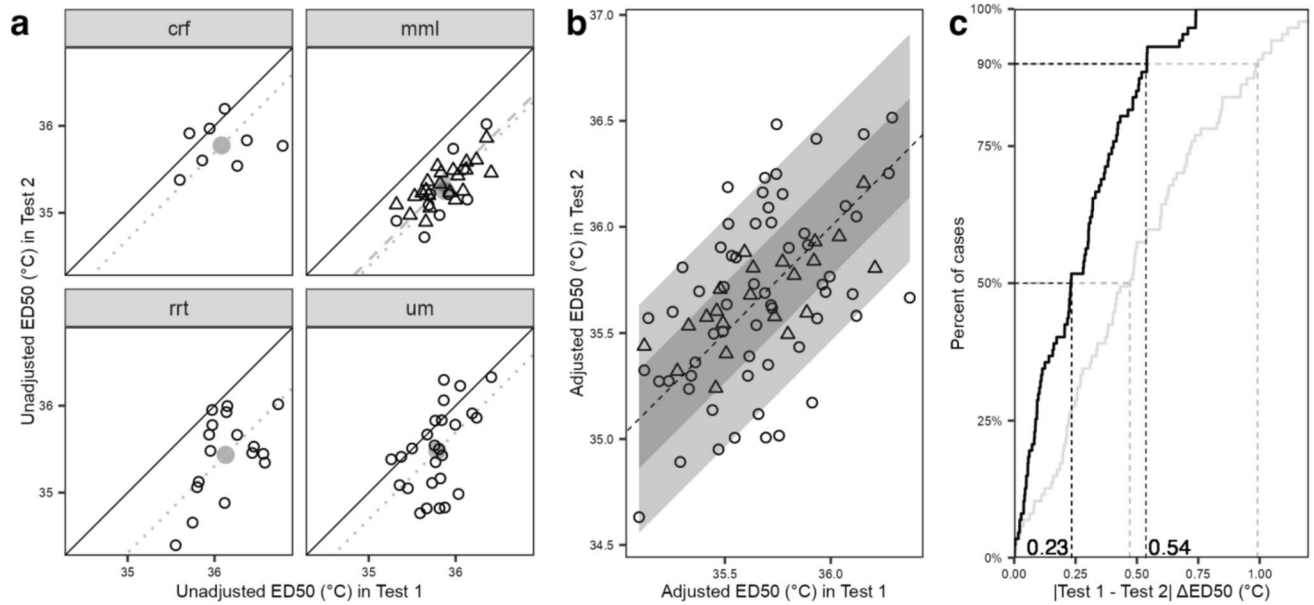


**Fig. 4** Test—retest reliability of repeated CBASS assays conducted on the same coral genets over time. ED50 values for each genet in test 1 (October 2020) and test 2 (September 2021 [circles] or June 2020 [triangles]) are shown in (**a**) for each nursery, with mean values for each nursery—test combination plotted as filled gray points. Solid diagonal lines indicate a line of identity, while broken lines pass through the mean values for each nursery and test date (dotted line = September 2021, dashed line = June 2020). Panel (**b**) shows adjusted ED50 values (subtracting variance due to nursery and test date, i.e. residuals from broken lines in panel (**a**)), with shading indicating the adjusted test—retest difference in 50% and 90% of cases. Panel (**c**) shows the empirical cumulative distribution of test–retest differences using adjusted (black line) or unadjusted (gray line) ED50 values, with annotations for the threshold differences under which 50% and 90% of cases occur

the probability that retesting random pairs of corals resolved them in the same rank order, based on how different they were in the first test. When comparing two individuals, only those whose adjusted ED50 values differed by > 0.93 °C were reliably resolved (i.e., 90% of the time) in the same rank order by a CBASS retest; unadjusted ED50 values had to differ by > 1.18 °C for the same confidence (Fig. 5). Groups of 3 corals were resolved in the same rank order in 90% of retests if their mean adjusted ED50 from the initial test was > 0.54 °C, as were groups of 10 corals that differ by > 0.29 °C. With unadjusted values, groups of 3 and 10 corals needed to differ by at least 0.64 °C and 0.35 °C, respectively, for 90% retest reproducibility (Fig. 5).

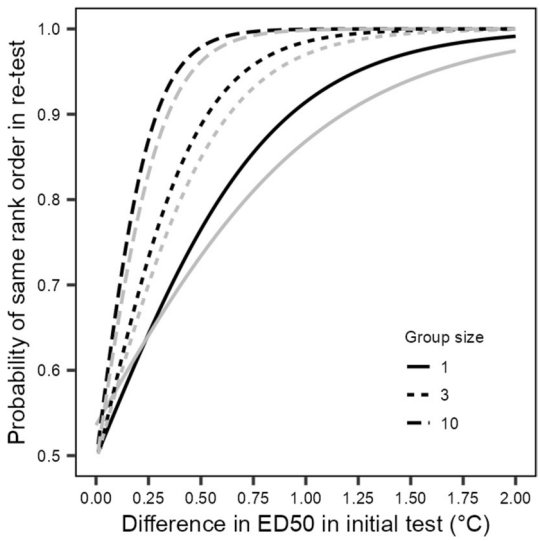


**Fig. 5** Probability of finding same rank order between coral individuals and groups in CBASS retest. Lines represent fitted values for binomial GLMs for comparing groups of N corals (1, 3, and 10 shown) using adjusted (black lines) and unadjusted (gray lines) ED50s

## Discussion

Here we investigated the use of CBASS as a diagnostic tool to rapidly quantify and compare coral heat tolerance thresholds for use in research and restoration. It is critical that thresholds derived from such a tool are 1) representative of bleaching resistance during natural marine heatwaves, and 2) reproducible and comparable across studies. We present evidence supporting both of these criteria, validating the utility of CBASS in identifying heat-tolerant corals, while

also quantifying its limitations in this context to guide future applications for research and restoration.

First, we demonstrate that CBASS-derived heat tolerance thresholds are highly predictive of performance in an 8-day classic heat stress experiment: *A. pulchra* colonies in Mo'orea that performed better in the rapid heat stress assay also performed better under longer-term heat stress. This finding builds on similar evidence from corals in American Samoa (Morikawa and Palumbi 2019) and the Red Sea (Evensen et al. 2021; Voolstra et al. 2020), indicating that CBASS produces equivalent information on individuals' relative heat tolerance in hours, making it a much more efficient approach for determining heat tolerance thresholds for corals with high throughput (up to ~ 40 colonies per day (Cunning et al. 2021; Evensen et al. 2023)). Most importantly, this finding establishes environmental and ecological relevance by confirming rapid acute heat stress assays can predict performance under longer-term stress resembling natural marine heatwaves.

However, marine heatwaves vary greatly in the magnitude and duration of heat stress accumulation, measured as Degree Heating Weeks (DHW) (Leggat et al. 2022), and these differences may affect the degree to which bleaching outcomes reflect CBASS-derived heat tolerance thresholds. For example, here we show that *A. pulchra* colonies' tolerances to 2.6 DHW over 8 days (in our 'classic' experiment) were strongly predicted by their CBASS ED50s (Fig. 3). Similarly, (Voolstra et al. 2020) found that tolerance to 2.34 DHWs over 8 days was correlated with CBASS ED50s for *Stylophora pistillata* colonies. In contrast, (Klepac et al. 2024) found that responses of *A. cervicornis* colonies to 1.13–1.43 DHWs over 11–14 days were not correlated with CBASS ED50s, which could reflect the lower heat stress accumulation and/or longer duration of the study. Indeed, we found that a significant relationship was not detected until sufficient heat stress accumulated in our classic experiment (Fig. S3). CBASS-derived thresholds may also become irrelevant in heatwaves so extreme as to cause bleaching and mortality of most or all corals, such as what occurred in 2023 for Caribbean acroporids that experienced > 20 DHWs (Hoegh-Guldberg et al. 2023); Kenkel et al. in review). Therefore, CBASS-derived heat tolerance thresholds may best predict differential coral responses to moderate heatwaves that fall near the average of individual tolerances.

Variation in heat tolerance thresholds may reflect fixed (i.e., genotypic) differences among corals, and effects of the environment, which influences corals' acclimatory state (Drury and Lirman 2021; Fitt et al. 2001; Fuller et al. 2020). Indeed, we found consistent shifts in heat tolerance thresholds when the same *A. cervicornis* genets were retested in different months and years, reflecting acclimatization to changing in situ environments (time and place explained 33.5% of ED50 variance). However, the relative differences and rank order of heat tolerance thresholds among individuals were largely maintained (Fig. 4b), suggesting there are also strong fixed differences (coral genet explained 51.4% of ED50 variance). This evidence for both genotypic and environmental effects underscores the importance of controlling for environmental factors when utilizing CBASS to identify heat-tolerant corals. To achieve this, comparing corals at the same time and place with identical environmental histories is ideal. When this is not possible, or when comparing corals across sites, then environmental parameters should serve as covariates in downstream analysis (e.g. (Marzonie et al. 2023)). Alternatively, a sufficient number of individuals should be tested from each site, so that site means can be used to adjust for variation due to environment (assuming individuals belong to the same population). This latter approach was taken here for *A. cervicornis*, with adjusted ED50s now reflecting a combination of genotypic and biological differences, and experimental and measurement error.

To assess these sources of variation, and test the overall reproducibility of CBASS-derived heat tolerance thresholds for individual corals, we compared adjusted ED50s of the same genotypes tested and retested up to a year apart. We found ED50s were reproduced within 0.23 °C in 50% of cases, and within 0.54 °C in 90% of cases. These margins confirm reproducibility at the individual level, but indicate there is still substantial additional variation that could be due to methodology or biology. Temporal changes in physiological and/or epigenetic states may vary among corals and alter the thermal tolerance phenotypes and resulting rank orders measured at any given time, though the magnitude of such effects is unknown (but could be effectively tested using CBASS). Methodological variation may include both experimental and measurement error, which could be amplified by any differences in experimental design or systems such as lighting, water flow, and temperature control, highlighting the importance of assay standardization to enable comparisons across studies (Grottoli et al. 2021; Nielsen et al. 2022; Evensen et al. 2023). However, even minimized experimental and measurement error still substantially limits the ability to make comparisons between individuals based on a single test.

Ideally, replicate assays would be performed on each individual to make comparisons more powerful, yet practical constraints on time and coral biomass may make single assays the only viable option, especially when testing many individuals is a competing goal. Therefore, it is critical to evaluate the robustness of comparing heat tolerance between corals based on single CBASS assays. We found that small differences between two corals are likely to reflect random error and are not reproducible; two individuals' adjusted ED50s had to differ by at least 0.93 °C to have a 90% probability of retesting in the same rank order. With unadjusted

ED50s (i.e., when environmental variation is not controlled for), this difference was even larger (1.18 °C). Such margins may span most of the ED50 range for a population (e.g., Florida *A. cervicornis* with an ED50 standard deviation of 0.38 °C; (Cunning et al. 2021)), meaning that only the top and bottom 5–10% of individuals can be confidently differentiated in a pairwise manner. While this may be a sufficient achievement in many cases, the finer-scale differences between the majority of individuals would remain indistinguishable from random error without further replicated tests.

However, the power to differentiate heat-tolerant corals increases significantly when looking at groups instead of individuals. Groups of 3 corals whose average ED50 differed by 0.54 °C were resolved in the same rank order by retests in 90% of cases, and for groups of 10 corals this margin reduced to 0.29 °C. Confidence in a retest producing the same ranking increased to 99% when groups of 10 corals differed by 0.60 °C (Fig. 5). In this study, the top 10 and bottom 10 *A. cervicornis* differed by 0.99 °C, translating to a 99.95% probability of retesting in the same rank order (indeed, they retested with a 0.72 °C difference). These examples demonstrate that CBASS can powerfully and reproducibly identify sets of corals with differential heat tolerance from a population. Whether this level of reproducibility extends to other coral species with varying life histories and morphologies (e.g., flat vs. branching, which could affect light exposure and responses in CBASS assays) should be prioritized in future work.

Collectively, our experiments across two *Acropora* species validate CBASS as an effective tool in providing standardized, individual-level diagnostics of coral heat tolerance that are ecologically relevant. We show that CBASS-derived heat tolerance thresholds were strongly predictive of individual-level performance during longer-term heat stress resembling a natural marine heatwave. And by retesting the same individuals with CBASS, we show these heat tolerance thresholds are reproducible, though there are limitations on their resolving power. For example, a single CBASS assay cannot distinguish fine-scale differences between individual corals from experimental error or environmental effects (especially when comparing across sites); however, it can powerfully identify groups of corals with larger differences in heat tolerance (e.g., top and bottom performers), especially when environmental variation is controlled for. This information is critical for researchers and practitioners utilizing CBASS, as it provides guidance on the level of variability that can be expected when measuring heat tolerance thresholds, particularly in branching Acroporids, and the level of confidence that comparisons of these thresholds reflect true biological differences in heat tolerance. These findings are pivotal for decision-making in high throughput screening scenarios that aim to identify heat-tolerant corals for propagation, genetic screening, selective breeding, or other restoration activities and interventions. Ultimately, such decisions and interventions may play an essential role in the survival and restoration of many coral populations in warming oceans, and CBASS can be a critical tool to inform these actions.

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**Data availability** All data and analysis scripts are available at github.com/jrcunning/CBASS_methods and archived at Zenodo (https://doi.org/10.5281/zenodo.13886873).

**Declarations**

**Conflict of interest** No competing interests declared.

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