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BOLD AWARD

IRON AVAILABILITY MODULATES THE RESPONSE OF ENDOSYMBIOTIC DINOFLAGELLATES TO HEAT STRESS[1]

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Warming and nutrient limitation are stressors known to weaken the health of microalgae. In situations of stress, access to energy reserves can minimize physiological damage. Because of its widespread requirements in biochemical processes, iron is an important trace metal, especially for photosynthetic organisms. Lowered iron availability in oceans experiencing rising temperatures may contribute to the thermal sensitivity of reef-building corals, which rely on mutualisms with dinoflagellates to survive. To test the influence of iron concentration on thermal sensitivity, the physiological responses of cultured symbiotic dinoflagellates (genus *Breviolum*; family Symbiodiniaceae) were evaluated when exposed to increasing temperatures (26 to 30°C) and iron concentrations ranging from replete (500 pM Fe') to limiting (50 pM Fe') under a diurnal light cycle with saturating radiance. Declines in photosynthetic efficiency at elevated temperatures indicated sensitivity to heat stress. Furthermore, five times the amount of iron was needed to reach exponential growth during heat stress (50 pM Fe′ at 26–28°C vs. 250 pM Fe′ at 30°C). In treatments where exponential growth was reached, *Breviolum psygmophilum* grew faster than *B. minutum*, possibly due to greater cellular contents of iron and other trace metals. The metal composition of *B. psygmophilum* shifted only at the highest temperature (30°C), whereas changes in *B. minutum* were observed at lower temperatures (28°C). The influence of iron availability in modulating each alga's response to thermal stress suggests the importance of trace metals to the health of coral-algal mutualisms. Ultimately, a greater ability to acquire scarce metals may improve the tolerance of corals to physiological stressors and contribute to the differences in performance associated with hosting one symbiont species over another.

Key index words: ***Breviolum*; iron concentration; micronutrients; photophysiology; Symbiodiniaceae; trace metal quotas**

***Abbreviations*: HR-ICPMS, high-resolution—inductively coupled plasma mass spectrometry; FIRe, Fluorescence Induction and Relaxation fluorometer; F_v/F_m, PSII photochemical efficiency; σPSII, PSII effective absorption cross sections; p, connectivity factor; τ_{QA}, electron re-oxidation rate of Quinone/Quencher A; DFA, discriminant function analysis; KW, Kruskal-Wallis**

Iron and other trace metals are essential for primary productivity in the ocean (Martin and

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Fitzwater 1988, Kolber et al. 1994). Continued ocean warming may significantly reduce iron solubility and availability, which could compromise the ability of microalgae to meet metabolic demands and result in loss of physiological integrity (Liu and Millero 2002, Hoffmann et al. 2012, Hutchins and Boyd 2016). Moreover, higher temperatures lead to increased rates of metabolism (Grégoire et al. 2017, Baker et al. 2018) and a greater dependency on iron-requiring processes which function to maintain photosynthesis, chlorophyll synthesis, macronutrient assimilation, and the detoxification of oxygen radicals to prevent cellular damage (Raven et al. 1999, Lin et al. 2001, Wolfe-Simon et al. 2005, Sunda 2012). Thus, coping with thermal stress depends in part on access to sufficient iron concentrations for physiological maintenance.

Photosynthetic dinoflagellates have among the highest iron requirements of microalgae (Brand et al. 1983, Sunda and Huntsman 1995, Ho et al. 2003, Rodriguez et al. 2016, Reich et al. 2020). Traits associated with mixotrophic lifestyles may account for why many dinoflagellates have higher iron needs relative to other phytoplankton groups (Stoecker 1999). The processes of photosynthesis, phagocytosis, and prey digestion involve numerous biochemical pathways that require iron and other trace metals (Stossel 1974, Raven et al. 1999, Behrenfeld and Milligan 2013). Additionally, the common occurrence of dinoflagellates in mostly coastal waters may relax selection pressures for economizing iron use relative to other open-ocean microalgae (Sunda and Huntsman 1995, Botebol et al. 2017). Thus, decreases in iron supply as the oceans warm may substantially impact the physiology of free-living dinoflagellates as well as those living in mutualisms with corals (Ferrier-Pagès et al. 2001, Shick et al. 2011, Hoffmann et al. 2012, Hutchins and Boyd 2016).

Climate change jeopardizes the health of reef-building corals by negatively impacting their relationship with mutualistic dinoflagellates from the family Symbiodiniaceae (Warner et al. 1999, Baker 2003, Cziesielski et al. 2019). Arguably, the stability of coral reef ecosystems rests on the continued function of mutualisms between corals and their dinoflagellate endosymbionts (family Symbiodiniaceae). The physiological response of these endosymbiotic dinoflagellates to increasing thermal stress may dictate the long-term persistence of coral communities and the ecosystems they construct (Brown 1997, Loya et al. 2001, Sampayo et al. 2008, Hoadley et al. 2019). Broad ranges of physiological differences are documented among species within and between genera of Symbiodiniaceae (Hennige et al. 2009, Díaz-Almeyda et al. 2017, Goyen et al. 2017, Grégoire et al. 2017, Mansour et al. 2018). The physiological characteristics that facilitate stress tolerance, are in part modulated by nutrient availability (Béraud et al. 2013, Wiedenmann et al. 2013, Ezzat et al. 2016, Rosset et al. 2017, Courtial et al. 2018, Ferrier-Pagès et al. 2018). Access to greater concentrations of nutrients, including macronutrients and trace metals, improves acclimation ability, which may substantially raise the ability of a species to cope with high temperatures (Rosset et al. 2017, Thomas et al. 2017, Ferrier-Pagès et al. 2018, Andrew et al. 2019, Aranguren-Gassis et al. 2019).

Nitrogen and phosphorus are scarce in most places where reef corals thrive (Odum and Odum 1955). Furthermore, nutrient limited (nitrogen, phosphorus) corals exhibit greater sensitivities to temperature stress (Béraud et al. 2013, Ezzat et al. 2016, Rosset et al. 2017, Courtial et al. 2018); whereas animals with greater nutrient reserves or access to exogenous sources are more likely to survive episodes of severe thermal stress (Loya et al. 2001, Shick et al. 2011, Aichelman et al. 2016, Ferrier-Pagès et al. 2018). While most research into the effect of nutrients on physiological acclimation and thermal stress have focused on the importance of macronutrients (Béraud et al. 2013, Wiedenmann et al. 2013, Courtial et al. 2018), considerably less is known about the importance of iron (and other trace metal micronutrients), which also is found at low concentrations in coral reef ecosystems (Menzel and Ryther 1961, Entsch et al. 1983, Sakka et al. 1999, Chase et al. 2006, Caputi et al. 2019).

Iron limitation may significantly affect the physiological function of a coral's endosymbionts, which, as dinoflagellates, often require more trace metals than other microalgae (Rodriguez et al. 2016, Reich et al. 2020, Yang et al. 2020). Retaining sufficient intracellular reserves or the ability to acquire iron rapidly may improve a reef building coral's ability to meet the metabolic demands imposed by high temperatures (Shick et al. 2011, Parkinson et al. 2015, Levin et al. 2016, Ferrier-Pagès et al. 2018). Given the vital role iron plays in biochemical processes, enhanced concentrations of this metal presumably impart a physiological benefit to the symbiont by allowing it to better acclimate to higher temperatures (Shick et al. 2011, Andrew et al. 2019). However, different symbiont species have distinct metal demands (Reich et al. 2020), which, depending on the availability of iron, will differently affect the response of each species to physiological stressors.

The experimental manipulation of cell cultures allows for detailed evaluation of how trace metal concentrations alter physiological performance of the algal symbiont. We used cultures of two species in the genus *Breviolum* that exist in symbiosis with cnidarians to explore how iron availability alters the response of tropical (*Breviolum minutum*) and temperate (*B. psygmophilum*) species to thermal stress (Thornhill et al. 2008, LaJeunesse et al. 2012). These experiments seek to understand how iron availability and differences in metal uptake

influence the capacity of dinoflagellate endosymbionts to acclimate to increasing temperatures. Ultimately, low iron availability may further destabilize their mutualisms with reef-building corals by exhausting the symbiont's capacity to meet the physiological demands of temperature stress.

MATERIALS AND METHODS

Culture maintenance. In order to effectively manipulate and maintain trace metal concentrations, all experimentation took place in laboratories at Academia Sinica equipped with class 100 trace metal clean facilities (Rodriguez et al. 2016, Rodriguez and Ho 2017, 2018, Reich et al. 2020). Cultures of *Breviolum minutum* (strain rt-002/ CCMP 2460) and *B. psygmophilum* strain (MAC-Pur.P.flex/ CCMP 3573) were maintained in modified L1 media at high iron concentrations (250 nM total dissolved Fe) and temperatures (26°C) for 14 months prior to experimentation (see below; Guillard and Hargraves 1993). The iron concentration (250 nM total dissolved Fe) was selected because it provides sufficient iron for reaching maximum growth rates (Rodriguez et al. 2016, Reich et al. 2020). Stock solutions were maintained in 250 mL polycarbonate bottles on a 12:12 h light:dark cycle with light intensities at 650 ± 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ and at ambient temperature (26°C). Culturing *Breviolum* spp. at saturating light levels prevented low-light acclimation and enabled comparison with previous research (Rodriguez et al. 2016, Rodriguez and Ho 2017, 2018, Reich et al. 2020). All seawater was filtered using 0.2 μm (Polycap 75, Whatman) filters and was passed through a Chelex® 100 resin at a rate of 2.5 mL · min^{-1} to remove background trace metals. Lastly, seawater was filter-sterilized using 0.22 μm Millipore filters (Ho et al. 2003, Rodriguez et al. 2016, Reich et al. 2020).

Acclimation to treatment conditions. Culture conditions were derived from previous work which determined sufficient nutrient concentrations for Symbiodiniaceae growth (Rodriguez et al. 2016, Rodriguez and Ho 2017, 2018, Reich et al. 2020). *Breviolum* spp. cultures were exposed to a full factorial set of iron concentrations (10, 50, 100 nM total dissolved Fe) and temperatures (26, 28, 30°C). This led to 18 total treatments and 54 culture bottles ($n = 3$ per treatment). The modified L1 culture media also included initial concentrations of nitrate (800 μM), phosphate (50 μM), B-vitamins, trace metals (10 nM Mn, Zn, Co, Cu, Ni). $FeCl_3.6H_2O$ (Fe^{3+}) was used for iron enrichment (Morel et al. 1979, Ho et al. 2003, Rodriguez et al. 2016). The vitamin B mixture consisted of 300 nM thiamine, 2.1 nM biotin, and 0.40 nM cyanocobalamin. To control biometal availability, 20 μM of trace metal clean grade EDTA was added to each 1 L SteriCap polycarbonate bottle. With the addition of EDTA 50, 250, and 500 pM Fe′ are bioavailable, which corresponded to the total dissolved iron concentrations 10, 50, and 100 nM Fe, respectively (Rodriguez et al. 2016). Hereafter, the iron concentrations will be referred to by their inorganic or bioavailable (Fe′) concentrations so that they correspond to other Symbiodiniaceae culture experiments in literature (Rodriguez et al. 2016, Rodriguez and Ho 2017, 2018, Reich et al. 2020).

To acclimate the stock cultures to the appropriate iron concentration, each treatment was maintained at the respective iron concentration for one month at ambient (26°C) temperatures. To acclimate the stock cultures to higher temperatures, the stock culture was ramped at a rate of $1°C \cdot d^{-1}$ until the target temperature was reached and then it was held at said temperature (28 or 30°C) for at least 12 d prior to experimentation. All treatments used for experimentation started from stock *Breviolum* spp. exposed to the aforementioned temperature*iron conditions at maximum cell density. The length of the (stock culture) acclimation period provided sufficient time to reach maximum cell densities and stationary phase without observing declines in cell pigmentation. All treatments were run with triplicate replicates with starting cell densities between 1,100–1,500 cells · mL^{-1}. After inoculation, growth was measured every other day during the light phase of the light:dark cycle until cultures reached stationary phase. Cell density and volume were enumerated using a Beckman Coulter Counter Multisizer 3 with a 100 μm aperture tube that quantified particles from 2.5–30 μm. During mid-exponential phase, *Breviolum* spp. cells were aliquoted in the trace metal clean room for the analyses described below.

Determination of pH. The pH of undiluted cells from each treatment was quantified using a Thermoscientific Orion 4 Star Plus probe at several growth stages: lag (before exponential growth at day 2), log (during exponential growth; on the same day samples were collected to measure nutrient contents and evaluated for photophysiology), and stationary phase (after exponential growth). Filtered sea water without culture medium added was used as a blank.

Determination of intracellular metal content (quotas), PON/POC content, pigment profiles, and total lipid content. During mid-exponential phase, *Breviolum* spp. cells were collected for determination of intracellular phosphorus and metal quotas (iron, zinc, copper, cobalt, nickel, manganese) using High-Resolution—Inductively Coupled Plasma Mass Spectrometry (HR-ICPMS, Element XR, Thermo Scientific). For each triplicate replicate, around ten million cells were harvested onto acid-washed 25 mm (2 μm pore size) Millipore TTTP polycarbonate filters during the light phase of the light:dark cycle. Cells were quickly rinsed with ultrapure Milli-Q water three times to remove seawater residue and were subsequently digested in 50% nitric acid for HR-ICPMS preparation. Metal and phosphorus content data was normalized to cell count (pg metal or phosphorus · $cell^{-1}$). The details of the HR-ICPMS analytical precision, accuracy, and detection limits for microalgal samples were described in the studies by Ho et al. (2003) and Ho (2013).

Aliquots of roughly ten million *Breviolum* spp. cells were collected for determining intracellular Organic Carbon (C) and Organic Nitrogen (N) concentrations. Cells were harvested on 25 mm (0.45 μm pore size) Whatman GFF filters and stored at −80°C until they were analyzed using the EA Elemental Analyzer 2400 CHNS/O Series II. POC/PON filters were baked at 400°C prior to usage. Carbon and nitrogen concentrations were blank corrected and normalized to cell count (pg C or N · $cell^{-1}$).

Aliquots of roughly ten million cells of *Breviolum* spp. were collected for determination of chlorophyll *a* pigment content. Cells were harvested on 25 mm (0.45 μm pore size) Whatman GFF filters and stored at −80°C until they were analyzed using High-Performance Liquid Chromatography (Shimadzu model SIL-20AC). Chlorophyll *a* content was normalized to cell count (pg chl *a* · $cell^{-1}$).

A final aliquot of roughly ten million *Breviolum* spp. cells were collected for determining total lipid content. Aliquots were flash-frozen and stored at −80°C. Sample aliquots were placed in a freeze drier for 4 h. Dried samples then underwent a modified Folch method extraction to estimate lipid levels in each treatment, which used chloroform, methanol, and 0.88% NaCl solution (Folch et al. 1957) to estimate total lipid content (mg lipid · $cell^{-1}$). Briefly, samples were gently agitated for two h in 8:4 (by volume) chloroform to methanol. Following this incubation period 0.88% NaCl was added, maintaining the following ratio 8:4:3 (by volume) chloroform, methanol, NaCl. The addition of NaCl solution facilitates the

separation of the chloroform and methanol/water phases, while also removing any water-soluble contaminants from the chloroform phase. The methanol/water phase was then removed, and the chloroform phase was dried under a steady N_2 stream until samples were dried. The lipid content was then gravimetrically quantified using an analytical balance (Mettler Toledo).

Determination of photosynthetic physiology. Aliquots of undiluted *Breviolum* spp. were taken during mid-exponential phase for analysis of photosynthetic measurements using a 2005 Satlantic FIRe (Fluorescence Induction and Relaxation) fluorometer (Halifax, Nova Scotia, Canada; Gorbunov and Falkowski 2004). Culture aliquots were poured inside the clean room and FIRe measurements were done outside of the clean room. All aliquots were dark-acclimated for 30 min prior to measurements. For each treatment, blanks consisting of the supernatant flow through from undiluted culture media poured over a 2 uM TTTP Millipore polycarbonate filter were measured using the same parameters as the experimental samples. *Breviolum* spp. were exposed to default settings including blue LED light, Gain: 400, Sample delay: 1000, Number samples: 50, STF: 120, STRP: 40, STRI: 60, MTF: 50, MTRP: 50, MTRI: 100. Blank-corrected results were generated using FIRePRO software.

The following photophysiological parameters were measured to assess the effect of temperature and iron availability on cell health and function: PSII (photosystem II) photochemical efficiency (F_v/F_m), PSII effective absorption cross sections (σPSII, sigma), connectivity factor (p), and electron re-oxidation rate of Quinone/Quencher A (Q_A; Tau, τ_{QA}). PSII photochemical efficiency (F_v/F_m) is the ratio of variable fluorescence to maximum fluorescence and represents the maximum photochemical efficiency of PSII in the dark (Kolber and Falkowski 1993, Kolber et al. 1994, Baker 2008). Sigma (σPSII) denotes the functional absorption of cross section for light that is used for photochemistry ($Å^2 \cdot \text{quanta}^{-1}$; Kolber and Falkowski 1993, Hennige et al. 2009). The connectivity factor (p) is the probability (from 0–1) of energy transfer from a closed PSII reaction center to an open PSII reaction center (Kolber and Falkowski 1993). Lastly, τ_{QA} is a time constant for re-oxidation of the quinone (Q_A) in the PSII reaction center (Kolber and Falkowski 1993, Behrenfeld and Milligan 2013, Kalaji et al. 2014). Because the effect of a 30-min dark acclimation was not measured, our analyses and interpretations correspond to comparisons between treatments (i.e., ambient temperature versus heat stress; high versus low iron concentrations).

Statistics. All statistical analyses were run in R v3.5.2 (R Core Team 2016). The specific growth rate of each treatment was calculated ($\frac{\Delta \ln(\text{cell density})}{\Delta \text{time } (d)}$; $\mu \cdot d^{-1}$). For trace metal content data, outliers were systematically removed within each treatment group using Mahalanobis square distance. A discriminant function analysis (DFA, MASS package) was used to determine the continuous response variables (i.e., iron, zinc, copper, other trace metal contents) that are most effective in distinguishing independent variables (i.e., treatment groups; Ripley 2002, Poulsen and French 2008, Ripley et al. 2013, Venables and Ripley 2013). The output of DFA also includes jack-knifing (cross-validation) to determine the proportion of correctly classified samples to each set of independent variables (i.e., species identity, iron concentration, temperature) and which treatment groups (independent variables) had samples that were misclassified with one another (Ripley 2002, Poulsen and French 2008, Ripley et al. 2013, Venables and Ripley 2013).

For clarification on a univariate scale, a Kruskal-Wallis test was used to determine differences in mean intracellular metal quotas, photosynthesis parameters, chlorophyll *a* pigment, and lipid content using the PMCMR package (Pohlert 2014). Subsequently, a Pairwise Test for Multiple Comparisons of Mean Rank Sums (Dunn's Test) was used as a post hoc test to determine differences between treatment groups using a Benjamini-Hochberg p-adjustment to account for false discovery rate (Benjamini and Hochberg 1995). Data were visualized using ggplot2 v2.2.1, ggpubr v0.1.6, and cowplot v0.8.0 (Wickham 2009, Wilke 2016). Bivariate and multivariate results for all metal content normalizations can be found in the supplement. All data and code are publicly available on github https://github.com/hgreich/ironXtemp.

RESULTS

Iron availability dictates Breviolum *spp. growth under thermal stress.* The specific growth rates for both *Breviolum* spp. were unaffected when grown at ambient (26°C) and moderately (28°C) warmer temperatures. Rates of growth slowed at 30°C in media with high and medium iron concentrations (Fig. 1). Cell proliferation experienced its most substantial decline when grown in low iron media (50 pM Fe′) while exposed to temperatures of 30°C (Fig. 1). For this treatment, the specific growth rates for *B. minutum* and *B. psygmophilum* decreased by 86 and 83% percent (respectively) relative to controls (grown at 30°C but with sufficient iron; Fig. 1). At all treatments, *Breviolum psygmophilum* specific growth rates were 12–52% higher than *B. minutum* (Fig. 1).

Stages in *Breviolum* spp. growth in culture corresponded to changes in pH of the culture media (Fig. S1 in the Supporting Information). When freshly inoculated batch cultures are at lag phase,

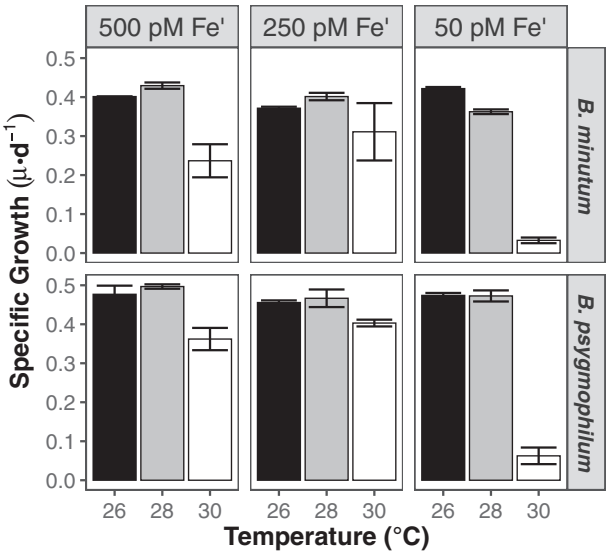

FIG. 1. Specific growth rate ($\mu \cdot d^{-1}$) of *Breviolum* spp. grown in different iron concentrations and temperatures. At higher iron concentrations, each species can withstand high temperatures, but cannot survive at 30°C when grown with low iron concentrations (50 pM Fe′). *Breviolum psygmophilum* grew 12–52% faster than *B. minutum* at all conditions. Bars represent the average of $n = 3$ replicates per treatment. Error bars represent ± standard deviation.

the culture media is below pH 8.0 (Fig. S1). At the stage of exponential cell growth in cultures with high and moderate iron concentrations, pH (measured during daylight) increased to ~ 8.5–10. These pH values remained high during stationary phase. For cells grown at 30°C, pH values were significantly lower and consistent with reduced rates of growth and lower cell densities (Fig. S1). In the low-iron high-temperature treatment, pH values did not change between lag, log, and stationary phases reflecting a lack of cell proliferation (Fig. S1).

Physiological responses to temperature stress under iron limitation. The photophysiological responses to moderate and high temperature treatments while grown at different iron availabilities differed for each species (Fig. 2). On average, PSII photochemical efficiency (F_v/F_m; KW chi-squared$_1$ = 10.4, $P < 0.01$), and PSII (photosystem) cross-section absorption (σPSII, sigma; KW chi-squared$_1$ = 30.5, $P < 0.01$) were significantly higher in *Breviolum psygmophilum* compared to *B. minutum* (Fig. 2). When exposed to low iron concentrations and high temperatures, the electron re-oxidation rate of Q_A (quencher/quinone pool A, Tau, τ_{QA}) decreased for both *B. minutum* (KW chi-squared$_7$ = 18.4, $P = 0.01$) and *B. psygmophilum* (KW chi-squared$_8$ = 21.7, $P < 0.01$; Fig. 2). Additionally, these conditions resulted in a decrease of PSII photochemical efficiency for both species but more so for *B. minutum* (KW chi-squared$_7$ = 19.4, $P < 0.01$) than *B. psygmophilum* (KW chi-squared$_7$ = 14.8, $P = 0.04$; Fig. 2).

Chlorophyll *a* content did not differ between the two species (Fig. S2 in the Supporting Information). Exposure to low iron concentrations resulted in nominal decreases of *Breviolum minutum* chlorophyll *a* content and had little effect on *B. psygmophilum* (Fig. S2). Contrastingly, chlorophyll *a* content of *B. psygmophilum* (KW chi-squared$_2$ = 10.7, $P < 0.01$) and *B. minutum* (KW chi-squared$_2$ = 6.1, $P = 0.047$) decreased significantly at high temperatures (30°C; Fig. S2). Neither species demonstrated significant changes in total lipid content in response to either stressor (Fig. S3 in the Supporting Information).

Cellular content of trace metals influenced by temperature and iron availability. Simultaneous exposure to different iron concentrations and elevated temperatures resulted in changes in trace metal contents, metal: Phosphorus ratios, and major nutrients unique to each species (Fig. 3, Figs. S4-S6 in the Supporting Information; Table 1; Appendix S1 in the Supporting Information). DFA output revealed species-specific thresholds of heat tolerance (Fig. 3). Specifically, *Breviolum minutum* exhibited a "heat-stressed" trace metal profile at a lower temperature (28°C) relative to *B. psygmophilum* (30°C; Table S1 in the Supporting Information; Fig. 3). The DFA infrequently misclassified samples grown at 26°C with those grown at 30°C (<10% for both species; Table S1). However, when exposed to moderately elevated temperature (28°C), *B. minutum* metal profiles more closely resembled high (30°C) temperature counterparts which was revealed by incorrect classification between the temperature treatment groups (88.2% correctly classified at 28°C, 60% at 30°C; Table S1; Fig. 3). Contrastingly, the DFA misclassified *B. psygmophilum* trace metal content at moderate (28°C) and ambient (26°C) temperature conspecifics (68.8% correctly classified at 26°C, 64.7% at 28°C; Table S1; Fig. 3). The

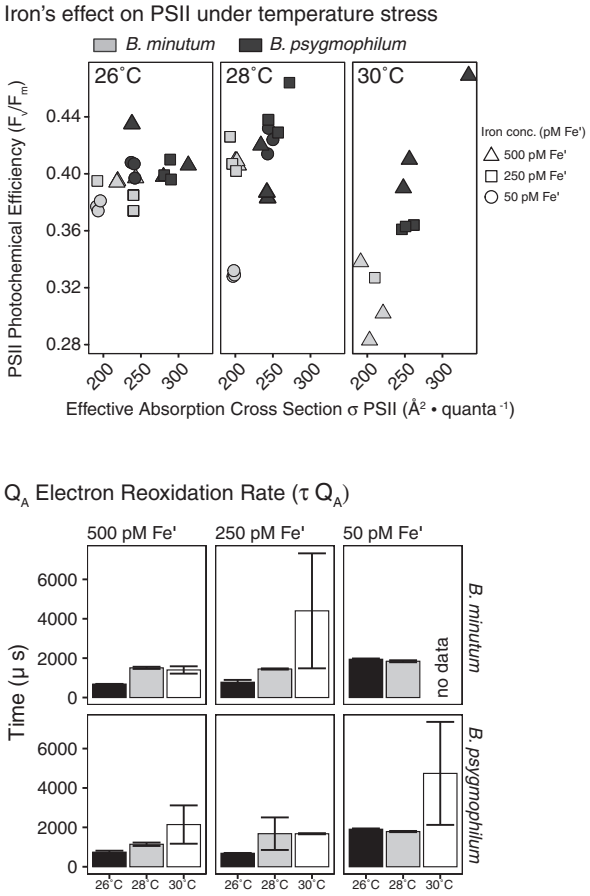

FIG. 2. The effect of iron concentrations on photophysiology while under thermal stress. (A) Relationship between Photosystem (PS) II photochemical efficiency (F_v/F_m) and PSII cross-section absorption (σPSII, sigma) for *Breviolum minutum* (grey points) and *Breviolum psygmophilum* (black points). The different iron concentrations are denoted by point shape. Exposure to higher temperatures resulted in a decrease of PSII (F_v/F_m) photochemical efficiency for both species but more so for *B. minutum* (KW chi-squared$_7$ = 19.4, $P < 0.01$) than *B. psygmophilum* (KW chi-squared$_7$ = 14.8, $P = 0.04$). Specifically, *B. minutum* PSII photochemical efficiency began declining at a lower temperature (28°C) and experienced more severe reductions at the highest temperature (30°C) than *B. psygmophilum*. *Breviolum psygmophilum* cross-section absorption (σPSII) was larger than *B. minutum* at all conditions (KW chi-squared$_1$ = 30.5, $P < 0.01$). (B) Electron re-oxidation rate of Q_A (τ_{QA}, Tau) increases in with high temperature and low iron exposure for both *B. minutum* (KW chi-squared$_7$ = 18.4, $P = 0.01$) and *B. psygmophilum* (KW chi-squared$_8$ = 21.7, $P < 0.01$). Longer Q_A re-oxidation rates (τ_{QA}) reflect compromised photosynthetic ability. Bars represent the average of $n = 3$ replicates per treatment. Error bars represent ± standard deviation.

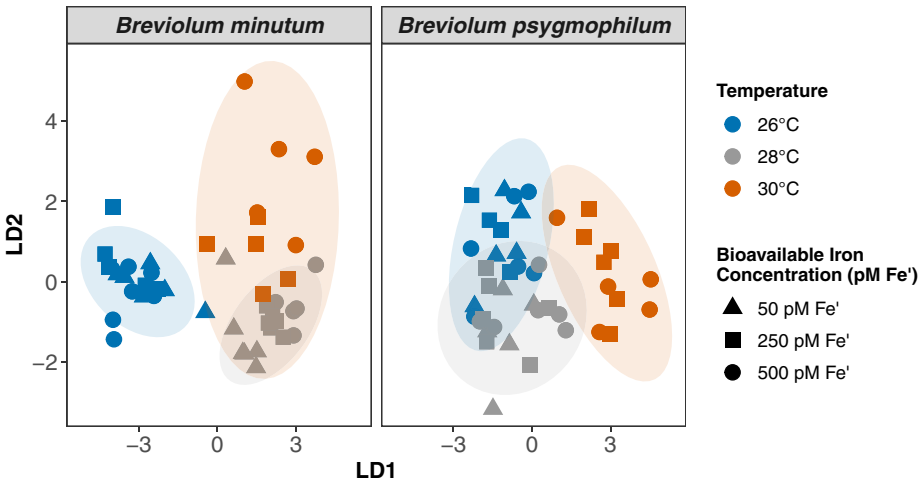

FIG. 3. A Discriminant function analysis (DFA) of metal concentrations (pg metal · cell^{-1}) exhibits how clustering patterns distinctive to (A) *Breviolum minutum* and (B) *B. psygmophilum* are correspond to different temperature thresholds. Alterations of *B. minutum* metal contents reflected heat stress at 28 and 30°C. In contrast, significant shifts of *B. psygmophilum* in metal content did not occur until temperatures reached 30°C. The temperature thresholds associated with the two species were reflected in the success of their classification rates. High levels of misclassification occurred for *B. psygmophilum* samples exposed to lower temperatures (68.8% correctly classified at 26°C, 64.7% at 28°C) whereas 91% of samples exposed to the highest temperature (30°C) were correctly classified. Contrastingly, 94% of *B. minutum* samples exposed to the lowest temperature (26°C) were correctly classified whereas misclassification of conspecifics exposed to higher temperatures was more frequent (88.2% correctly classified at 28°C, 60% at 30°C).

coefficients of linear discriminants for copper and cobalt were most important response variables for distinguishing *B. minutum* metal profiles at each temperature (Table S1). Similarly, the manganese and iron coefficients of linear discriminants were the most important for distinguishing *B. psygmophilum* trace metal profiles associated with each temperature (Table S1).

Exposure to low (50 pM Fe′) iron concentrations resulted in alterations of the metal profiles and metal: Phosphorus ratios, and major nutrient contents of the two species (Table 1; Figs. S4-S6; Appendix S1). The DFA correctly classified the metal profiles of both species at low iron concentrations (Table S2 in the Supporting Information). Contrastingly, some samples exposed to the medium and replete iron concentrations were misclassified with one another (59.5% average correct classification rate for *Breviolum minutum,* 82% for *B. psygmophilum*; Table S2). The coefficients of linear discriminants driving changes in *B. minutum* trace metal content at low iron concentrations were primarily copper and manganese (Table S2). The cobalt and copper coefficients of linear discriminants were the most important response variables in characterizing changes in *B. psygmophilum* trace metal profile at low iron concentrations (Table S2).

DISCUSSION

Metals required for protein synthesis and enzyme activity are essential for numerous metabolic processes, including physiological acclimation. Therefore, iron availability should modulate the threshold at which symbiotic dinoflagellates can acclimate to thermal stress (Fig. 1; Shick et al. 2011, Ferrier-Pagès et al. 2018). Indeed, low iron availability significantly decreased cell proliferation under high temperature conditions by raising the thermal sensitivities of *Breviolum minutum* and *B. psygmophilum* (Fig. 1). Moreover, alterations in cellular compositions of trace metals correspond to differences in heat tolerance between each species (Fig. 3). The physiological consequences of trace metal limitation as it relates to the stability of coral-dinoflagellate mutualisms during episodes of severe warming are discussed below.

Higher temperatures increase the need for iron. Iron availability is a limiting micronutrient in meeting the metabolic demands imposed by stressful temperatures (Figs. 1, S1; Kudo et al. 2000, Shick et al. 2011, Andrew et al. 2019). Warming temperatures can raise a dinoflagellate's cell division rate, but at a certain point further increases rapidly diminishes the cell's ability to proliferate (Figs. 1, S1; Grégoire et al. 2017, Baker et al. 2018, Mansour et al. 2018, Rodriguez and Ho 2018, Andrew et al. 2019). As temperatures become increasingly stressful, larger energy investments in acclimation slow and may arrest other cellular processes. Excessively high temperatures intensify rates of respiration and cellular repair, which require the synthesis of more proteins including many enzymes that require metal co-factors (Kudo et al. 2000, Toseland et al. 2013). Such physiologically demanding conditions increase the need to upregulate metalloenzymes dedicated to the detoxification of oxygen radicals (i.e., superoxide dismutase) to prevent widespread cellular

TABLE 1. Mean (± standard deviation) specific growth ($\mu \cdot d^{-1}$) and intracellular organic carbon (pg C · cell^{-1}), organic nitrogen (pg N · cell^{-1}), phosphorus (pg P · cell^{-1}), and trace metal contents (fg metal · cell^{-1}) of *Breviolum minutum* and *B. psygmophilum* grown at different iron concentrations and temperatures. Cells failed to grow in low iron (50 pM Fe′) medium at the 30°C, resulting in insufficient biomass for elemental measurements.

Temp	pM Fe′	Species	Specific Growth	Carbon (pg · cell^{-1})	Nitrogen (pg · cell^{-1})	Phosphorus (pg · cell^{-1})	Iron (fg · cell^{-1})	Cobalt (fg · cell^{-1})	Copper (fg · cell^{-1})	Manganese (fg · cell^{-1})	Nickel (fg · cell^{-1})	Zinc (fg · cell^{-1})
26°C	50	*B. minutum*	0.42 ± 0.0	14.5 ± 0.3	2.34 ± 0.0	0.57 ± 0.1	3.08 ± 0.2	0.15 ± 0.05	0.07 ± 0.01	1.2 ± 0.31	0.04 ± 0.01	0.44 ± 0.12
	250	*B. minutum*	0.37 ± 0.0	12.9 ± 0.8	2.12 ± 0.1	0.42 ± 0.1	4.6 ± 1.1	0.12 ± 0.02	0.07 ± 0.02	0.75 ± 0.15	0.19 ± 0.03	0.24 ± 0.06
	500	*B. minutum*	0.4 ± 0.0	13.1 ± 0.6	2.12 ± 0.1	0.33 ± 0.0	3.65 ± 0.2	0.10 ± 0.01	0.07 ± 0.01	0.55 ± 0.05	0.18 ± 0.01	0.38 ± 0.16
	50	*B. psygmophilum*	0.47 ± 0.01	13.9 ± 1.9	2.41 ± 0.3	0.2 ± 0.0	2.23 ± 0.3	0.11 ± 0.03	0.20 ± 0.07	0.57 ± 0.10	0.04 ± 0.01	0.46 ± 0.17
	250	*B. psygmophilum*	0.46 ± 0.01	29.8 ± 1.0	4.14 ± 0.2	0.47 ± 0.0	5.83 ± 0.4	0.37 ± 0.02	0.16 ± 0.01	1.75 ± 0.11	0.27 ± 0.04	0.46 ± 0.03
	500	*B. psygmophilum*	0.48 ± 0.02	18 ± 0.9	3.15 ± 0.2	0.3 ± 0.1	4.35 ± 0.9	0.14 ± 0.05	0.11 ± 0.02	0.84 ± 0.25	0.15 ± 0.04	0.68 ± 0.65
	50	*B. minutum*	0.36 ± 0.01	11.3 ± 1.6	1.76 ± 0.2	0.43 ± 0.1	2.61 ± 0.6	0.08 ± 0.01	0.07 ± 0.01	0.61 ± 0.10	0.03 ± 0.00	0.23 ± 0.03
	250	*B. minutum*	0.4 ± 0.01	14.5 ± 0.4	2.07 ± 0.0	0.47 ± 0.1	3.99 ± 0.7	0.10 ± 0.02	0.08 ± 0.01	0.58 ± 0.09	0.03 ± 0.00	0.35 ± 0.15
	500	*B. minutum*	0.43 ± 0.01	14.6 ± 0.7	2.13 ± 0.1	0.51 ± 0.1	5.18 ± 0.5	0.12 ± 0.01	0.08 ± 0.01	0.54 ± 0.04	0.04 ± 0.00	0.36 ± 0.08
28°C	50	*B. psygmophilum*	0.47 ± 0.01	17.4 ± 3.8	2.55 ± 0.7	0.35 ± 0.1	3.25 ± 0.5	0.18 ± 0.04	0.23 ± 0.04	1.1 ± 0.23	0.05 ± 0.01	2.1 ± 1.4
	250	*B. psygmophilum*	0.47 ± 0.02	21.9 ± 1.0	3.1 ± 0.2	0.38 ± 0.0	4.7 ± 0.4	0.23 ± 0.03	0.22 ± 0.01	1.3 ± 0.23	0.18 ± 0.04	0.65 ± 0.04
	500	*B. psygmophilum*	0.5 ± 0.01	22.2 ± 0.1	3.2 ± 0.2	0.35 ± 0.1	5.41 ± 0.5	0.21 ± 0.02	0.17 ± 0.01	1.3 ± 0.15	0.12 ± 0.06	0.57 ± 0.18
	250	*B. minutum*	0.31 ± 0.07	11.1 ± 4.0	1.86 ± 0.3	0.62 ± 0.2	5.74 ± 0.8	0.13 ± 0.03	0.10 ± 0.04	0.74 ± 0.10	0.06 ± 0.01	0.35 ± 0.26
	500	*B. minutum*	0.26 ± 0.0	10.1 ± 0.9	1.85 ± 0.1	0.63 ± 0.1	6.58 ± 0.0	0.11 ± 0.04	0.10 ± 0.04	0.76 ± 0.09	0.06 ± 0.00	0.20 ± 0.00
	250	*B. psygmophilum*	0.4 ± 0.01	20.5 ± 10	3.03 ± 1.7	0.19 ± 0.0	4.42 ± 0.5	0.17 ± 0.05	0.13 ± 0.05	1.3 ± 0.29	0.05 ± 0.02	0.20 ± 0.10
	500	*B. psygmophilum*	0.36 ± 0.03	13.7 ± 1.5	1.89 ± 0.3	0.18 ± 0.0	4.94 ± 0.2	0.16 ± 0.03	0.12 ± 0.01	1.3 ± 0.12	0.05 ± 0.01	0.16 ± 0.04

damage (McGinty et al. 2012, Krueger et al. 2014, Levin et al. 2016, Goyen et al. 2017). Thus, increases in biochemical and cellular activities during thermal stress elevate the need for additional trace metal supply, especially iron (Kudo et al. 2000, Levin et al. 2016, Andrew et al. 2019).

As temperatures were raised to 30°C, reductions in cell proliferation was most severe when grown at low iron concentrations (50 pM Fe′; Fig. 1). The slowing of electron re-oxidation rates (Q_A turnover, τ_{QA}) revealed how these conditions compromised photosynthetic ability (Fig. 2). The flux of electrons re-oxidized by the plastoquinone pool (τ_{QA}) is a key metric for evaluating photosynthetic function under temperature stress and is regulated by a non-heme iron group (Raven et al. 1999, Suggett et al. 2015, Goyen et al. 2017). These decreases in electron transfer efficiency negatively affect energy production by photosystem II and likely contribute to declines in growth (Figs. 1, 2; Robison and Warner 2006, Goyen et al. 2017). The greater sensitivity to thermal stress with low iron availability emphasizes how external environmental factors, including trace metal concentrations, modulate an algal cell's ability to cope with thermal instability (Figs. 1, 2, S1; Shick et al. 2011, Andrew et al. 2019). Future experiments on their mutualisms with corals should parse how, or to what degree, low iron availability affects processes requiring metalloenzymes beyond photosynthesis, including major nutrient assimilation and anti-oxidant activity.

Nutrient access influenced differences in physiology between species. Access to sufficient nutrient reserves is critical for maintaining homeostasis and meeting the metabolic demands of stressful conditions. For example, the macronutrient phosphorus is important for maintaining energy reserves (ATP), building nucleic acids, critical to the composition of cell plasma membranes, and an essential element for many other cellular and biochemical processes (Schachtman et al. 1998, Orchard et al. 2010, Ferrier-Pagès et al. 2016). When limited, it diminishes the response of many biochemical and physiological pathways that are relied upon to cope with severe thermal stress (Ezzat et al. 2016). It was therefore surprising that a more "heat sensitive" strain (*Breviolum minutum*) possessed larger phosphorus reserves than the "heat resistant" strain (*B. psygmophilum*; Table 1; Figs. 1, 2, S6). Despite having higher phosphorus concentrations, *B. minutum* incurred greater photodamage (i.e., larger reductions in PSII photochemical efficiency, F_v/F_m) and precipitous declines in cell proliferation (Figs. 1, 2). It is possible that much of the resident phosphorus was unavailable for use, perhaps bound to, or utilized in, the cell's wall matrices, or allocated to an ongoing stress response such as nucleic acid repair rather than pathways involved in cell proliferation (Figs. 1, S6; Donk et al. 1997, Rodriguez-Casariego et al. 2018). The sensitivity of *B. minutum*

to heat stress might also be attributed to the low metal-to-phosphorus ratios measured in its cells (Fig. S5). Future experiments should determine how different balances of metal and phosphorus reserves result in allocation to various cellular processes.

Along with macronutrients, sufficient supplies of essential trace metals are equally important for maintain physiological homeostasis and cell proliferation. The more "heat resistant" strain, *Breviolum psygmophilum* had faster cell proliferation rates, experienced less photodamage at high temperatures (i.e., less pronounced declines in PSII photochemical efficiency, F_v/F_m), and maintained larger reserves of trace metals (e.g., copper, cobalt, zinc, manganese, nickel) despite its lower phosphorus contents (Table 1; Figs. 1-3, S4–S6). Enhanced physiological capabilities might be modulated by greater concentrations of some metals present in *B. psygmophilum* relative to *B. minutum* (Figs. 3, S4, S5; Reich et al. 2020). For example, larger manganese supplies found in *B. psygmophilum* may better support the elevated PSII photochemical efficiency (F_v/F_m) and light absorption (σPSII) while exposed to high temperatures when grown under low iron concentrations (Figs. 2, S4, S5; Kolber et al. 1994, Raven et al. 1999, Goyen et al. 2017, Reich et al. 2020). If differences in the composition and abundance of trace metals correspond to the innate attributes that differentiate species, then those able to more actively acquire metals, or maintain greater reserves, can improve their capacity to acclimate to thermal stress (Figs. 1-3; Parkinson et al. 2015, Levin et al. 2016). Ultimately, access to iron (and other metals) during stressful conditions may rival the importance of phosphorus as a critical nutrient for enhancing thermal tolerance (Wiedenmann et al. 2013, Rosset et al. 2017, Ezzat et al. 2019).

Shifts in metal composition differed between species in response to temperature increases. Cellular compositions of trace metals appeared sensitive to increases in temperature, which may influence the physiological responses among different dinoflagellates species (Fig. 3). The temperature threshold at which metal contents shifted differed between the two species and corresponded to their heat resistance (Figs. 1-3). The metal profile of the more "heat sensitive" *Breviolum minutum* changed when grown at medium (28°C) and high temperatures (30°C) relative to control temperatures (26°C; Figs. 3; S4). In contrast, significant shifts in the metal contents of the "heat resistant" strain of *B. psygmophilum* did not occur until temperatures were raised above 28°C (Fig. 3). These unique changes in metal contents may relate to different sensitivities to thermal stress (Figs. 1-3; Thornhill et al. 2008, Klueter et al. 2017, Mansour et al. 2018, Kishimoto et al. 2020). *Breviolum psygmophilum* proliferates more rapidly in culture, has higher photosynthetic efficiency, withstands a wider temperature range and persists across a larger range of latitudes than *B. minutum* (Figs. 1 and 2; Thornhill et al. 2008, LaJeunesse et al. 2012, Grégoire et al. 2017, Grupstra et al. 2017, Mansour et al. 2018). Perhaps maintaining sufficient intracellular metal (i.e., iron) content during episodes of rapid warming is a cause, or consequence, of greater physiological integrity (Figs. 1-3; Shick et al. 2011, Parkinson et al. 2015, Ferrier-Pagès et al. 2018). Greater shifts in the content of cellular metals, relative to life at normal temperatures, may therefore be indicative of greater thermal stress and thus provides another metric with which to compare thermally sensitive and tolerant species (Fig. 3). Ultimately, these observations highlight how maintaining sufficient intracellular metal content may correspond to thermal stress tolerance (Figs. 1-3).

CONCLUSIONS

With sufficient iron, reef-forming corals are able to better withstand thermal stress (Fig. 1; Shick et al. 2011). However, inadequate iron access may have adverse consequences for the stability of their obligate mutualisms with dinoflagellates. Iron limitation significantly lowered the thermal tolerances of two cultured symbionts (Figs. 1, 2). Sensitivity to metal limitation and its effect on biochemical pathways may point to more subtle, yet critical, factors influencing stress responses among dinoflagellates in general and symbiotic species in particular. Decreases in trace metal solubility (availability) alongside ocean warming (Hoffmann et al. 2012, Hutchins and Boyd, 2016) may further destabilize coral-dinoflagellate mutualisms leading to increases in "coral bleaching" (Shick et al. 2011, Ferrier-Pagès et al. 2018). Very little is known about how life inside a host's cell membrane alters the trace metal availability to resident symbionts and its effect on their thermal tolerances. The development of biomarkers for assessing metal transport and storage, as well as applying high-resolution imaging techniques (i.e., nanoSIMs) to characterize subcellular trace metal localization and transport, combined with gene expression studies, may contribute further to our understanding of how micronutrients are important to a symbiont's physiology (Parkinson et al. 2015, Decelle et al. 2020, Li et al. 2020). As Earth's oceans continue to warm, having a better understanding of how trace metals influence the stability of coral-dinoflagellate partnerships is imperative.

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AUTHOR CONTRIBUTIONS

HGR, TCL, TYH, and IBR conceptualized the study. TYH, TCL, HGR, and DWK provided funding. TCL, TYH, and IBR provided graduate student mentorship. HGR and IBR performed algal culture experiments. TYH, TCL, and DWK provided reagents. WT and YC performed HR-ICPMS, pigment, and particulate organic carbon and nitrogen analyses. EFK and DWK provided lipid content data. HGR conducted photophysiology, statistical analyses, and wrote the first draft of the manuscript. HGR and TCL led further writing of the manuscript. TYH, DWK, IBR and EFK contributed to editing the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Change in pH through lag (before exponential growth at day 2), log (during exponential growth), and stationary phase (after exponential growth) for *Breviolum* spp. exposed to different iron concentrations and temperature.

Figure S2. *Breviolum* spp. chlorophyll a content (pg chlorophyll $a \cdot \text{cell}^{-1}$) decreases at high temperature (30°C) which reflects declines in photosynthetic ability during temperature stress.

Figure S3. Changes in *Breviolum* spp. total lipid content (mg lipid $\cdot$ cell^{-1}) when exposed to different iron concentrations and temperatures.

Figure S4. Changes in *Breviolum* spp. intracellular iron, manganese, zinc, cobalt, copper and nickel content (pg metal $\cdot$ cell^{-1}) when exposed to different iron concentrations and temperatures.

Figure S5. Changes in *Breviolum* spp. intracellular iron, manganese, zinc, cobalt, copper and nickel content normalized to Phosphorus (pg metal:P $\cdot$ cell^{-1}) when exposed to different iron concentrations and temperatures.

Figure S6. Changes in *Breviolum* spp. intracellular organic carbon (pg C $\cdot$ cell^{-1}), organic nitrogen (pg N $\cdot$ cell^{-1}), and phosphorus (pg P $\cdot$ cell^{-1}) concentrations when exposed to different iron concentrations and temperatures.

Table S1. Output for the Discriminant Function Analyses (DFA) of the metal concentrations (pg metal $\cdot$ cell^{-1}) *Breviolum* spp. when exposed to different temperatures.

Table S2. Output for the Discriminant Function Analyses (DFA) of the metal concentrations (pg metal $\cdot$ cell^{-1}) *Breviolum* spp. when exposed to different iron concentrations.

Appendix S1. Supplemental results.