Molecular, physiological, and organismal-level effects of thermal stress in corkscrew sea anemones *Bartholomea annulata*

by

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Abstract

Changing ocean dynamics in the form of increasing seawater temperature are having drastic effects on the symbiotic relationships of coral reef cnidarians, causing them to bleach and die at increasing frequencies. Most research on thermotolerance in cnidarians revolves around the reef-building stony corals; much less information is available on the physiological effects of non-calcifying reef cnidarians such as the soft-bodied sea anemones. Despite the central ecological role of reef-building corals, it is important also to determine potential future effects of thermal stress on reef anemones, as these cnidarians may have major effects on tropical marine communities. On Caribbean coral reefs, corkscrew anemones *Bartholomea annulata* serve as hosts for Pederson's cleaner shrimp, and provide visual cues for client fishes who approach them as cleaning stations to have their ectoparasites removed. This study aims to address gaps in our knowledge concerning whole-organismal processes, physiological functions, and molecular enzyme kinetics of corkscrew anemones in response to seawater temperature variability. The responses of this anemone species to temperature stress are described both under field conditions in the Florida Keys, and under laboratory conditions in aquarium cultures. In the field, the body size and microalgal characteristics of *B. annulata* varied with temperature, in that they were maximized at moderate temperatures, and exhibited reduced values indicating sublethal effects at extreme temperatures. Surface seawater temperature varied widely between winter (14°C) and summer (34°C) over 2 years in all 3 regions of the Keys examined. Chlorophyll concentration and microalgal abundance in anemone tentacles were significantly lower during summer in the Lower Keys than winter in the Middle and Upper Keys, indicating bleaching during the warmest season at the southernmost sites. Laboratory experiments revealed that anemone survival, body size, and microalgal abundance all decreased significantly at extreme temperatures, but increased

within the optimal range of 22 - 25°C. Some individuals exposed to sublethal temperatures (18 or 32°C) later recovered after return to optimum, but those at < 14 or > 34°C all bleached and died. The effect of acclimation temperature was also studied at the physiological level and revealed that anemone respiration rate increases nearly linearly with temperature up to 32°C, while photosynthetic rate of symbiotic algae is maximal at only 22°C. This difference leads to a lower P:R ratio at higher temperatures, indicating a net loss of energy when compared to photosynthetic output. Temperature also significantly affected the potential metabolic activity measured via electron transport system activity, indicating that anemones at moderate reef temperatures are already operating at near maximal cellular levels.

I conclude that these sea anemones experience seasonal thermal stress especially in the Lower Florida Keys during summer, when temperatures approach their upper lethal limit. Unbalanced energy budgets due to respiratory rate outpacing photosynthetic output at high temperatures may induce bleaching and subsequent loss of additional mutualistic benefits, leaving the anemone completely reliant on heterotrophic food sources. Florida Keys populations of *B. annulata* likely will decline under climate change, with consequences for reef fish health due to concurrent loss of their associated cleaner shrimp. Coral reef managers should focus on identifying at risk populations based on annual temperature regime and limit commercial harvest of this species during physiologically stressful summer months.

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RMR	resting metabolic rate
NPR	net photosynthetic rate
PAR	photosynthetically active radiation
PMA	potential metabolic activity
TCSA	tentacle crown surface area
P:R	ratio of photosynthesis to respiration
ETS	electron transport system
TPC	thermal performance curve
MO_2	oxygen consumption rate
PO_2	oxygen partial pressure
T_{opt}	optimum temperature for performance

Chapter 1

Sea anemones in a changing environment

Climate change effects in marine environments

For the past 420,000 years, atmospheric carbon dioxide has fluctuated between 190-300 ppm (IPCC 2001), but today concentrations stand at nearly 400 ppm, mainly due to the burning of fossil fuels and cement production (Global Greenhouse Gas Reference Network 2016). The rate of CO_2 emissions continues to grow each year by approximately 1%, but even at current levels, if anthropogenic emissions were to end immediately, it will take tens of thousands of years for levels to return to pre-industrial conditions (Raven et al. 2005). Since the beginning of the Industrial Revolution, the world's oceans have acted as a substantial carbon sink reservoir, absorbing around 50% of total CO_2 emissions. The role of the oceans as carbon sinks influences a variety of chemical, physical, biological and geological processes, many of which are important to marine organisms. It is projected that over the next 1,000 years, the role of the oceans will continue to grow in that they will absorb approximately 90% of the CO_2 released by anthropogenic activities (Archer et al. 1998). Major ecological changes are expected to arise from the large amounts of CO_2 that currently are being introduced into the oceans, including alteration of the marine carbon cycle, lowered pH of seawater causing ocean acidification, and increased seawater temperatures.

Increased water temperature is causing thermal stress in marine environments due to the effect of CO_2 on saltwater temperature during climate change. As greenhouse gases are trapped in the atmosphere, the ocean has acted as a sink not only for anthropogenically-generated carbon, but also for the excess surface heat that is a side effect of the trapped carbon, with an average

warming rate of 0.11°C per decade in the upper 75 meters of the water column (IPCC 2013). While this change does not seem drastic, even small temperature variations can have effects on a large scale, due to the vastness of the world's oceans. Sea level rise caused by thermal expansion, polar ice melt, and stronger and more frequent tropical storms, coupled with the enhanced thermally-based spread of marine diseases (Harvell et al. 2009) and spread of invasive species (Stachowicz et al. 2002) all are threats to marine organisms caused by increasing ocean temperatures. Yet another outcome of warming temperature is altered oceanic current patterns, which in turn can affect the surface temperatures of large land masses, nutrient circulation, and dispersal patterns of marine species (Hoegh-Guldberg and Bruno 2010). Even fundamental processes such as primary productivity are being impacted, with models predicting a 2-20% decline in global primary productivity by 2100 (Steinacher et al. 2010). Perhaps the most biologically-relevant effect of rising temperatures in marine systems is how the internal physiological processes of marine organisms are becoming altered. Cellular physiological functions such as rates of molecular diffusion and enzymatic reactions can influence patterns of organismal metabolism, growth, and mortality, in turn potentially changing population trends, so that consequences as large as altered ecosystem function may result from changing seawater temperatures. Changes in cellular respiration are susceptible to temperature fluctuation, potentially causing the energy needs of marine organisms to shift (Lopez-Urrutia et al. 2006; Paradis et al. 2019). Additionally, rising temperatures may affect reproductive processes through thermo-dependent sex determination in marine turtles (Bull and Vogt 1979), fishes (Ospina-Alvarez and Piferrer 2008), and copepods (Korpelainen 1990), thereby altering sex ratios. Range shifts may also occur, leading species to move further toward the poles to escape high temperatures (Perry et al. 2005). Some organisms may be able to acclimatize to higher

temperatures and changing environmental conditions, but others may be unable to adapt quickly enough, resulting in population declines and even extinction (Hoegh-Guldberg and Bruno 2010).

Ecological importance of coral reef cnidarians

As the effects of climate change cascade throughout the world's oceans, some of the most vulnerable marine environments are coral reefs. Coral reefs are among the most biodiverse and economically valuable ecosystems on the planet, supporting over 1 million species, and producing an estimated economic benefit of more than 29.8 billion USD per year (Cesar et al. 2003). Coral reefs provide coastal protection services, and support commercial fisheries and tourism industries for billions of people all over the world (Hoegh-Guldberg et al. 2007). Reef-building scleractinian corals create the reef framework that supports these enormous biodiversity and economic benefits, constructing complex three-dimensional habitats for the species that live on the reef or depend on it for survival. Major cnidarian reef-dwellers include the actinian sea anemones (Order Actiniaria), which are close relatives of the stony corals (Order Scleractinia). Sea anemones play important ecological roles on reefs by forming complex symbiotic relationships, serving as hubs of mutualistic networks that provide ecological benefits to individuals and entire communities (Huebner et al. 2019).

Most reef anemones host single-celled microalgae within their gastrodermal tissues. This endosymbiotic relationship, in which the anemone provides inorganic nutrients such as nitrogen to the photosynthetic algae, and the algae in turn release energy-rich compounds to the host, also forms the foundation of reef coral symbiosis (Cantrell et al. 2015). Of the 8 major clades of *Symbiodinium* (Clades A through H, as well as many sub-clades referred to as *Symbiodinium*

types; LaJeunesse 2002, Coffroth and Santos 2008), some occur exclusively within stony corals, but many also are found in sea anemones. In addition to algal endosymbionts, large reef anemones host a diverse array of obligate and facultative exosymbionts such as fish and crustaceans (Colombara et al. 2017). The familiar mutualistic relationship between sea anemones and anemonefish involves an intricate network of benefits such as fish waste excretions benefiting the microalgae populations through ammonia transfer (Roopin and Chadwick 2009), oxygenation of anemone tissues through fish movements (Szczeback et al. 2013), and protection of anemones from predation by their resident fish (Porat and Chadwick-Furman 2004). Additionally, some cleaner shrimp form obligate associations with coral reef anemones, using them as base stations for cleaning services that they provide to fishes. Anemones serve as visual cues to client fishes, which approach the cleaning stations to be rid of ectoparasites (Heubner and Chadwick 2012). Fishes that visit cleaning stations have greatly reduced parasite loads compared to those which do not, resulting in healthier fish populations (Becker and Grutter 2004). Cleaner organisms not only improve fish health, but contribute to fish biodiversity and prevent loss of important species from reefs, supporting the overall ecological condition of coral reefs (Grutter et al. 2003).

In addition to serving as hubs for cleaning services, anemones contribute to primary productivity at rates similar to those of marine algae. In some reef habitats, they can be among the top primary producers given adequate percent cover (Fitt et al. 1982). Furthermore, the presence of anemones on reefs can reduce algal recruitment and overgrowth via production of compounds for interspecific competition (Bak and Borsboom 1984). In terms of their direct usefulness to humans, many pharmaceutical products have been extracted from anemones for the treatment of disorders such as multiple sclerosis, autoimmune diseases, and even cancer (Linher-

Melville and Singh 2014; Chi et al. 2012; Beeton et al. 2011). Finally, sea anemones play a role in the multi-million dollar marine ornamental trade, with the market demand for many actinian anemones increasing each year (O'Reilly and Chadwick 2018). Due to their ecological and economic importance, it is imperative to understand the potentially complex effects of climate change on these unique reef cnidarians.

Effects of thermal stress on anemones and other reef cnidarians

Of the chemical and thermal changes in seawater that are occurring due to anthropogenic climate change, the increasingly wide temperature swings play a major role. Understanding both the upper and lower thermal limits of marine organisms is of value for determining how populations will be affected by rising or falling sea temperatures, and is important for successful management of populations (Portner 2001). For reef-building corals, the most dangerous threat of increasing temperature is the potential for bleaching, which occurs when the host experiences stress-related loss of endosymbiotic algae through expulsion. Excess heat appears to cause loss of photosynthetic function in the algae, due to photoinhibition and subsequent build-up of reactive oxygen species (Weis 2008), which represent a threat to the host and induce them to then expel their algal cells. The breakdown of this symbiotic relationship leads eventually to the death of the host, if it is not able to recover its algae quickly enough after the bleaching event, within a few days to weeks (Hoegh-Guldberg 1999). In addition to the effects of bleaching, increased temperatures impact coral sexual reproduction, larval settlement, and survival (Albright and Mason 2013; Ross et al. 2013; Paradis et al. 2019). While effects on corals have

been well-documented, much less information is available about how soft-bodied cnidarians such as sea anemones react to thermal stress.

Sea anemones, like all organisms, have thermal tolerance limits beyond which they cannot survive. Chomsky et al. (2004) found that high temperatures under laboratory conditions resulted in reduced biomass and shrinkage of intertidal Mediterranean sea anemones *Actinia equina*. In a field study of corkscrew anemones *Bartholomea annulata* in Florida, O'Reilly and Chadwick (2017) found that populations experienced the most rapid growth and lowest mortality during cool winter months and in northern habitats where seawater temperatures were ~ 24-27°C, in contrast to warm summer periods and southern habitats where temperatures were higher (30-35°C). In addition, O'Reilly et al. (2018) observed even slower growth and higher mortality in populations closer to the equator in the U.S. Virgin Islands, where they experienced temperatures of ~ 27-31°C (NOAA NDBC 2016). At temperatures outside the normal range for a given population, there is a risk of physiological stress, which may be expressed in sea anemones via increased metabolic rates such as higher oxygen consumption (Griffiths 1977; Walsh and Somero 1981), and reduced growth and regeneration (Reitzel et al. 2013). The thermal history of some cnidarians such as corals affects their respiration rates during thermal challenge (Castillo and Helmuth 2005). The respiration rates of temperate anemones are significantly higher during warm spring and summer months than in cool winter months (Verde and McCloskey 2007). However, little information is available on the thermal physiology of tropical reef anemones, including their lethal temperatures or optimal temperatures for growth.

Overview of dissertation chapters

This dissertation adds to the growing body of literature on the thermal biology of tropical marine invertebrates, by elucidating impacts of thermal stress on the major Caribbean reef anemone *Bartholomea annulata*. Experimental data are presented on effects of thermal stress on the growth and survival of the host and general characteristics of the symbiotic microalgae (Chapter 2), as well as on aspects of metabolic physiology and cellular performance (Chapter 3). These data contribute to understanding the basic biology of sea anemones, and also provide a scientific basis to support conservation management of this important coral reef species, by projecting impacts to populations under future climate change.

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Chapter 2

Effects of thermal stress on Caribbean sea anemones *Bartholomea annulata:* Survival, growth, and symbioses

Abstract

Thermal stress driven by climate change is causing widespread loss of tropical reef organisms, but thermotolerance levels remain unknown for many coral reef anemones. Corkscrew sea anemones *Bartholomea annulata* are important ecologically on Caribbean coral reefs, as hubs of mutualistic networks based on their associated cleaner shrimp. We hypothesized that characteristics of *B. annulata* vary with temperature in the Florida Keys, in that they function highest at moderate temperatures, and exhibit sublethal to lethal effects at both high and low extreme temperatures. The abundance and chlorophyll-a concentration of endosymbiotic microalgal cells in anemone tentacles were significantly lower during warm summer periods, indicating bleaching during the warmest season at the southernmost sites. Laboratory experiments revealed that anemone survival, body size, and microalgal abundance all decreased significantly at extreme temperatures, but increased within an optimal range. Individuals exposed to sublethal temperatures are able to recover from thermal stress. We conclude that these anemones experience seasonal thermal stress, primarily when temperatures approach the upper observed lethal limit determined by the laboratory study. Populations of *B. annulata* in the Florida Keys and elsewhere in the Caribbean likely will decline during anthropogenic climate change, with consequences for coral reef fish health due to concurrent loss of their associated cleaner shrimp.

Introduction

Global climate change is causing major impacts on the world's oceans, especially on coral reefs which are predicted to cease to exist as functional ecosystems by ~ 2050 (Heron et al. 2017; Hughes et al. 2017). Coral reefs comprise one of Earth's most biodiverse and economically valuable ecosystems, in that they support > 1 million species and produce almost $30 billion per year in economic benefits (Cesar et al. 2003). Physiological processes of coral reef species are severely altered by climate change induced temperature fluctuations (Lesser 2011, Sully et al. 2019. Some coral reef species may gradually acclimatize to climate change-induced higher temperatures, but others are likely to be unable to adapt quickly enough, resulting in population declines and even species extinctions (Hoegh-Guldberg and Bruno 2010). Understanding the thermal limits of reef species is important for determining how populations will respond to changes in seawater temperature, and provides a scientific basis for the management of populations during climate change (Portner 2001).

Most research on the effects of temperature variability on coral reefs revolves around the calcifying organisms that build reefs, in particular stony corals (Suggett et al. 2012, Paradis et al. 2019), but little is known about effects of thermal stress on close relatives of scleractinian corals, including the actinian sea anemones. Sea anemones are important ecologically on tropical reefs, in that they shelter both zooxanthellae (Cantrell et al. 2015) and a diverse array of obligate and facultative exosymbionts, namely fishes and crustaceans (Dixon et al. 2014; Colombara et al. 2017; Huebner et al. 2019). In the Caribbean Sea, reef anemones host obligate cleaner shrimp species which rid reef fishes of ectoparasites in this region, thereby enhancing the health of

fishes (Grutter et al. 2003; Huebner and Chadwick 2012). The presence of anemones on reefs additionally can reduce macroalgal recruitment and overgrowth via production of compounds for interspecific competition with algae (Bak and Borsboom 1984). Better understanding of the thermal limits of common coral reef anemones is needed, both to compare with those of stony corals, and to provide a scientific basis for their conservation management under climate change. Some information exists concerning the effects of thermal stress on growth and survival of sea anemones, but mostly for temperate or Indo-Pacific species. Thermal variability causes a variety of stress responses in temperate and tropical sea anemones including change in rates of diffusion and enzymatic reactions (Ainsworth et al. 2009, Putnam et al. 2013), oxygen consumption (Griffiths 1977, Walsh and Somero 1981), and growth and regeneration rates (Reitzel et al. 2013), clonal reproduction (Glon et al. 2018).

However, almost nothing is known about the thermotolerance of large coral reef anemones in the Caribbean Sea. In the Florida Keys, corkscrew sea anemones *Bartholomea annulata* are one of the most common anemones on Caribbean coral reefs, ranging widely from the Gulf of Mexico east to Bermuda and south to Brazil; they reproduce both sexually via broadcast spawning and asexually by budding (Colin 1978; Jennison 1981) to form small aggregations of polyps (Titus et al. 2017). These anemones are ecologically important as hosts to zooxanthellae belonging to *Symbiodinium* clades A and C (Grajales et al. 2016), as well as at least 6 species of ectosymbiotic crustaceans (Huebner et al. 2019). Especially important is their role as a major host to Pederson cleaner shrimp, which are the main crustacean cleaners of Caribbean reef fishes (Huebner and Chadwick 2012; Titus et al. 2019). Despite their ubiquitous presence and ecological importance in the Caribbean Sea, no experimental work has examined the thermotolerance of this anemone.

In the present study, we investigated effects of seawater temperature on the abundance, survival, and growth of corkscrew sea anemones *B. annulata*, and on characteristics of their zooxanthellae. We addressed 2 major hypotheses: (1) Body size and microalgal characteristics of field populations in the Florida Keys vary with ambient seawater temperature, both spatially among sites and temporally among seasons, and (2) survival and growth of laboratory individuals are highest at moderate coral reef seawater temperatures, with clear sublethal to lethal effects at temperature extremes. Our results have implications for the management of Caribbean reef ecosystems during global climate change, and for the commercial aquaculture of this species as an alternative to the collection of wild individuals for the ornamental aquarium trade.

Methods

Field observations

To address the first hypothesis that characteristics of field populations in the Florida Keys vary with ambient seawater temperature, both spatially among sites and temporally among seasons, we used previously-published data (1910-2019) and data collected during the present study (2017-2019). Three types and time periods of temperature data were compiled: (1) Annual variation over the past ~ 100 years (1910-2019) in air temperature at Key West, from information in the Applied Climate Information System maintained by NOAA Regional Climate Centers (http://xmacisrcc-acisorg/), as recorded daily at station Key West (Station ID 12858 WBAN), and then calculated as mean temperature for each year; (2) Monthly variation over the past 5 years (Jan 2014 - Sept 2019) in sea surface temperature at 1 weather buoy in each of 3

regions of the Keys (Lower, Middle, and Upper; NOAA NDBC 2019; http://www.ndbc.noaa.gov/; Fig. 2.1), as recorded hourly, and calculated as mean temperature each month; and (3) Seasonal variation over the past 2 years (2017-2019) in sea surface temperature at 6 sites in the Keys, as measured in person at 10 cm below sea surface, using a digital thermometer (Coralife Digital Thermometer, Coralife, Franklin, WI, USA) during the present study.

We also compiled published information about the abundance and body size of corkscrew sea anemones *Bartholomea annulata* from one-time surveys conducted prior to the present study, in March 2015 at 2 field sites in each of the same 3 regions of the Keys: Lower (Bowman's Channel and Cudjoe), Middle (Tiki Hut and Quarry), and Upper (Robbies and Indian Channel), and from 1 year of seasonal sampling each 3 months at the Cudjoe and Quarry sites (Mar 2014, Jun 2014, Sep 2014, Dec 2014, and Mar 2015; GPS headings given in O'Reilly and Chadwick 2017).

We then conducted field observations during the present study each 6 months for 2 years (5 sample periods: Sep 2017, Feb 2018, Sep 2018, Feb 2019, and Sep 2019; with some periods missing data due to storms; see below) at 2 field sites in each of the 3 main regions: Lower Keys (Cudjoe [24°39'00" N, 81°31'00" W] and Horseshoe Quarry [24°39'25" N, 81°18'10" W]); Middle Keys (Quarry [24°45'00" N, 80°58'48" W] and Fire Academy [24°44'59"N, 80°58'40"W]); and Upper Keys (Robbie's [24°52'48"N, 80°42'00" W] and Buttonwood Bay [25°6'05" N, 80°26'20" W];Fig. 2.1). Some of the sites surveyed here differed from those examined previously, because anemone abundances were no longer high enough for sampling at some previously-used sites. We selected sites for field observations during 2017-2019 based on the following criteria: they differed from each other in seawater temperature range (as recorded

by the above buoys; NOAA NDBC 2019), contained abundant individuals of *B. annulata*, and some of them had been examined previously (1 site in each region: Cudjoe, Quarry, and Robbie's; O'Reilly and Chadwick 2017). All field sites surveyed during the present study were adjacent to shore at 0.5 – 1.0 m depth, in habitats of rocky rubble, or coral and rubble interspersed with sand.

During 2017-2019, we determined seasonal variation in the characteristics of sea anemones and their zooxanthellae at each of the 6 field sites. Each site was examined ~ 1 month after the annual temperature minimum and maximum each year (which occurred in Aug and Jan, see Results); 1 month is the approximate period required for zooxanthellae to alter their characteristics in response to environmental changes (Roopin and Chadwick 2009, Cantrell et al. 2015). As such, all 6 sites were sampled during late winter (Feb) and late summer (Sep) each year. The following characteristics of *B. annulata* sea anemones were determined at each site during each sample period: abundance (not measured Sep 2017), body size, and zooxanthellae traits (cell abundance, mitotic index, and chlorophyll-a concentration). Anemone abundance was determined by deploying a 25-m transect parallel to shore in the area of maximal anemone abundance at each site. Then a random number generator app (Random Number Generator, AppleStore) was used to determine placement of ten 0.5x0.5m quadrats along the transect, on either the left or right side of the transect tape, and all *B. annulata* anemones were counted within each quadrat. Body size of 1 anemone within each of the 10 quadrats was measured as tentacle crown length (L) and width (W) for calculation of tentacle crown surface area (TCSA), using the equation for an oval: $(L/2*W/2)*\pi$ (Huebner et al. 2012; O'Reilly and Chadwick 2017). Each linear size measurement was taken using a plastic ruler to the nearest 1 mm. The ruler was placed as close as possible to the anemone without touching it, to avoid disturbing the

animal and causing body contraction (after Dixon et al. 2014). Additionally, to determine zooxanthellae characteristics, 3-5 tentacle tips (each ~ 2 – 3 cm length; Roopin et al. 2011) were removed from each measured anemone using scissors. Tentacle tips were transferred into a 2-ml plastic tube (Nalgene cryo-tube, Nalge Nunc International), transported to shore, and frozen in an acetone-dry ice bath within 30 minutes of collection. This method allowed accurate sampling of microalgal traits under field conditions; anemone tentacle samples can remain unfrozen for up to 30 minutes in the field without significant changes in their zooxanthellae (Rossi and Snyder 2001).

Tentacle samples remained frozen during transport to Auburn University within 1-2 days of collection, and then were placed in a -80°C freezer for up to 2 weeks prior to examination of zooxanthellae. Tentacle samples were thawed and weighed to the nearest 0.001 g using an electronic scale, homogenized in a micro-homogenizer, suspended in 1 ml of filtered sea water, and centrifuged for 5 minutes at 5000 rpm. The pellet was re-suspended in 1 ml of filtered seawater, with 0.5 ml of the resulting slurry used for zooxanthellae cell counts and 0.5 ml used for chlorophyll-a analysis (see below). A haemocytometer (Hausser Scientific, Horsham, PA, USA) was used to count individual zooxanthellae in each sample; the mean value of 5 counts was used to calculate the number of cells per gram wet mass of anemone tentacle (D'Elia and Cook 1988; Fitt and Cook 2001). Mitotic index of zooxanthellae was determined as the proportion of cells undergoing cell division at the time of sampling (Roopin et al. 2011) and calculated by dividing the total number of cells by the number of dividing cell couplets observed. Chlorophyll-a level per cell also was determined; 0.5 ml of the slurry was recentrifuged, the supernatant removed, and 1 ml of 90% acetone was added to the pellet, covered with aluminum foil, and maintained overnight at 4°C. Using a spectrophotometer (Thermo Electrical

Corporation Genesys 5), the absorbance of the sample was measured at 630 nm, 664 nm, 690 nm, and 750 nm using cuvettes, and 90% acetone as a blank between samples. Chlorophyll-a was calculated using the following equation: μgr chl-a ml-1=11.43(OD_{664}-OD_{750})-0.40(OD_{630}-OD_{750}) X acetone vol. (ml) (Roopin and Chadwick 2009).

Laboratory experiments

To address the second hypothesis that characteristics of laboratory individuals are optimized at moderate coral reef seawater temperatures, with clear sublethal to lethal effects at temperature extremes, we conducted laboratory experiments during March 2017 - March 2019. Individuals of *B. annulata* for laboratory experiments were obtained from commercial suppliers (KP Aquatics, Tavernier Key; Sea Critters, Marathon Key, Florida, USA). The anemones (body size range ~ 50-150 cm^2 TCSA) were cultured at Auburn University in 75 L tanks supplied with overhanging filters (Aqueon QuietFlow20; Aqueon), tank heaters (Eheim Jager TruTemp), and T5 florescent lights (AquaticLife Marquis Marine 24" Dual Lamp T5 HO). Conditions in the culture tanks mimicked those in the coral reef habitats occupied by this anemone: seawater temperature ~ 25 ± 1 °C, light:dark cycle 12:12 hrs, irradiance level ~ 60-90 μmol photons m^{-2} $s^{-1}$of photosynthetically active radiation (PAR), and low concentrations of dissolved nutrients. Individuals were fed thawed *Artemia* sp. (Frozen Brine Shrimp, San Francisco Bay Brand) 1x per week to satiation. Similar culture conditions have been used previously to support long-term growth of tropical sea anemones in the same laboratory (Roopin and Chadwick 2009, Huebner et al. 2012, Cantrell et al. 2015). All anemones were maintained under these conditions for > 1 month prior to use in experiments; they expanded their tentacles and either grew or maintained body size, indicating healthy condition.

Eight temperature treatments were applied (14, 16, 18, 22, 25, 28, 32, 34°C), spanning known seawater temperature range on Florida coral reefs (~ 16-32°C, Fig. 2.5), and including extreme temperatures at either end of this range, to determine the lethal temperature maximum and minimum for this species. Each anemone was assigned randomly to 1 of 8 treatment temperatures, with 2-3 tanks per treatment (24 treatment tanks total), and 4-7 individual anemones per tank (10-19 individuals per treatment x 8 treatments = 110 total individuals). Anemones were grouped in treatment tanks due to space constraints that prevented using a separate tank for each anemone, a design used in past studies (Chomsky et al. 2004, Roopin and Chadwick 2009). Each individual was maintained in a small glass bowl (~ 9 cm width x 4 cm height), allowing for individual identification of anemones.

The low temperature treatments were achieved using chillers (Chill Solutions CSXC-1 Thermoelectric Aquarium Chiller, and Prime Chiller Current with Ranco Electronic Temperature Control unit), and the high temperature treatments with aquarium heaters (Jager TruTemp, and Aqua Logic Digital Temperature Controller and heating element). Seawater temperature was changed 1°C per day from the 25°C culture temperature, for the treatments that required temperature change (after Chomsky et al. 2004), to mimic natural rates of temperature change on coral reefs (Griffiths 1977).

Each experimental treatment was applied for 6 weeks, followed by a 4-week recovery period when all tanks were returned to the culture temperature of 25°C (modified after Roopin and Chadwick 2009). Changes in sea anemone body size were determined weekly by measuring the linear dimensions of each polyp in terms of TCSA, similar to the assessment of body size at field sites (see above). Anemones were recorded as having died if they were observed to decompose in the tank, which can occur quickly over < 1 week (McMahon et al. 2017). To

assess changes in zooxanthellae, tentacle tips (2-3 cm length) were removed from a randomly-selected subset of anemones (N = 10 of the 10-19 individuals per treatment) every other week (0, 2, 4, and 6 weeks after start of treatments, 2 and 4 weeks after start of recovery). More limited sampling was conducted of zooxanthellae than of whole anemones, because the former is a labor-intensive process, and this sampling intensity is adequate to detect changes in zooxanthellae in response to environmental treatments (Roopin et al. 2011). Zooxanthellae traits (cell abundance per gram wet mass of anemone tentacle, mitotic index, and chlorophyll-a concentration per cell) were determined using the same methods as for field anemones (see above).

Statistical analysis

All statistical analyses were conducted using R Studio (R Core Team 2016). To determine the extent to which our field data supported the first hypothesis that characteristics of field populations in the Florida Keys vary with ambient seawater temperatures, both spatially among regions and temporally among seasons, we applied a linear model that treated temperature, period, and region as factors, and included an interaction variable between the three factors, to examine variation in 2 sea anemone characteristics (body size, abundance) and 3 zooxanthellae characteristics (cell abundance, mitotic index, chlorophyll-a level) among the 3 examined regions during 4 sample periods. Interaction between temperature, period, and region was found to be significant for all characteristics with the exception of mitotic index, so factors for those characteristics were analyzed separately using ANOVA followed by Tukey post-hoc pairwise comparisons.

To determine the extent to which our laboratory data supported the second hypothesis that characteristics of laboratory individuals are optimized at moderate coral reef seawater temperatures, with clear sublethal to lethal effects at temperature extremes, we also applied a linear model. The linear model treated both period (week) and temperature as categorical factors, and included an interaction factor between the two. It examined variation in one sea anemone characteristic (body size) and three zooxanthellae characteristics (cell abundance, mitotic index, chlorophyll-a level) among eight experimental temperatures during 10 weeks of laboratory experiments (both treatment and recovery periods). Interaction between temperature and period was found to be significant, so temperatures and periods were analyzed separately using ANOVA followed by Tukey post-hoc pairwise comparisons. To analyze variation in percent survival among the experimental temperatures and weeks, we applied a Pearson's chi-square test, and post-hoc pairwise nominal independence tests. Data are presented as means + one standard error (SE) unless otherwise indicated.

Results

Field observations

Long-term trends over the past century revealed a gradual increase in annual mean air temperature at Key West, with a minimum annual mean of 24.0°C in 1910, and a maximum of 26.8°C in 2019 (Fig. 2.1A). Over the past 5 years (2014-2019), the monthly mean surface seawater temperature as recorded by weather buoys varied widely on a seasonal basis in all 3 major regions of the Florida Keys, with a maximum each year in summer (Jul-Aug) and a minimum in winter (Jan; Fig. 2.1B). The most extreme mean monthly temperatures occurred

within the past 2 years, ranging from a low of 13.9°C in Jan 18 to a high of 34.9°C in Jun 19, while less extreme temperature variation characterized earlier years. Temperatures recorded by hand at each of 6 field sites during the present study (2017-2019) were within the range of those recorded by weather buoys, but were less extreme, from a low of 19.0°C during Feb 19 at the Quarry, to a high of 32.3 during Sep 19 at Buttonwood Bay (Fig. 2.1B).

In terms of spatial variation among regions of the Florida Keys, over the past 2 years when anemones were sampled (2017-2019), the seawater temperature as recorded by weather buoys was lowest during Jan 18 in the Upper Keys (13.9°C), moderate in the Middle Keys (15.6°C), and highest in the Lower Keys (17.3°C). Conversely, the temperature was highest in Aug 17 (just before the first fall sample period for anemones) also in the Upper Keys (34.4°C) slightly lower in the Middle Keys (34.1°C), and lowest in the Lower Keys (32.7°C ; NOAA NDBC 2017; Fig. 2.1B; see also O'Reilly and Chadwick 2017 for patterns during previous years). Seawater temperatures as recorded by hand showed similar variation among the 3 regions, and as mentioned above were within the range of monthly means calculated from the weather buoys. The two types of temperature measures were highly correlated (buoy-measured temperature = 0.991 hand-measured temperature + 0.515; $r^2 = 0.99$; N = 12 hand measurements), indicating that data from nearby NOAA buoys could be used to estimate seawater surface temperature as measured by hand at nearshore shallow sites year-round. Overall, the compiled temperature data revealed that Upper Keys sites experienced wide seawater temperature fluctuations during the 2 years of the present study (~ 14-34°C), and Lower Keys sites exhibited a slightly narrower range (~ 17-33°C).

The abundance of corkscrew sea anemones *Bartholomea annulata* over the past 4 years also varied widely among sites and seasons (Fig. 2.3A). Anemone abundances from previously

published observations during March 2015 were 0.35 individuals m^{-2} in the Lower Keys, 1.17 individuals m^{-2} in the Middle Keys, and 2.68 individuals m^{-2} in the Upper Keys (site locations differed slightly between O'Reilly and Chadwick 2017, and the present study; see Fig. 2.2). Only a few days after the first data collection period, Hurricane Irma passed directly over the southernmost site (Cudjoe; Fig. 2.2) as a Category 4 Hurricane on 10 Sep 2017. During the next sampling period in Feb 2018, anemones were absent from all field sites (0 individuals m^{-2}), except at Cudjoe where there were 2 anemones 25 m^{-2} (< 0.1 anemones m^{-2}). During the following sample period in Sept 2018, populations had recovered in the Lower Keys to 6.80 ± 1.69 individuals m^{-2} at Cudjoe, in the Middle Keys to 4.00 ± 1.33 individuals m^{-2} at the Quarry, and in the Upper Keys to 2.40 ± 1.77 individuals m^{-2} at Buttonwood Bay (Fig. 2.3A). However, 3 field sites (Horseshoe Quarry, Fire Academy, and Robbie's) remained without sea anemones (0 individuals m^{-2}), and did not regain populations of *B. annulata* during the present study. During Feb 2019, anemone abundances at the 3 sites that retained populations decreased substantially to only 2.40 ± 0.88 individuals m^{-2} at Cudjoe, 2.40 ± 1.22 at the Quarry, and 1.20 ± 0.85 individuals m^{-2} at Buttonwood Bay. During the final field survey in Sep 2019, abundance then increased again at all sites to 6.00 ± 1.49 at Cudjoe, 3.20 ± 0.99 at the Quarry, and 1.60 ± 0.65 individuals m^{-2} at Buttonwood Bay.

Statistical analysis indicated that anemone abundance varied significantly with both temperature and period, but not among regions of the Keys (Table 2.1A). When regions were grouped into periods, abundance was significantly higher during Sep 2018 than Mar 2015 or Feb 2019, but there were no significant differences between any other pairs of periods. When periods were separated by region, the Lower Keys contained significantly higher anemone abundance during Sep 2018 than either Mar 2015 or Feb 2019, similar to the whole-Keys pattern above, and

also had higher abundance during Sep 2019 than Mar 2015. In contrast, anemone abundance in the Middle and Upper Keys did not vary significantly among study periods (Supplemental Table 1A).

In contrast to anemone abundance, the body sizes of sea anemones varied significantly with all 3 variables examined: temperature, period, and region (Fig. 2.3B, 2.1B). When all periods were considered, body size was significantly larger in the Middle Keys than in both the Upper and Lower Keys throughout the study. When variation among regions of the Keys was analyzed separately for each period, body size did not differ among regions during Sep 2017, but it did in Sep 2018 with significantly smaller individuals in the Upper than Middle and Lower Keys, and the latter 2 regions not differing from each other. During Feb 2019, the Middle Keys had larger individuals than both the Lower and Upper Keys, which did not differ from each other. Then during the final sample period in Sep 2019, all 3 regions differed significantly from each other, with anemones in the Middle Keys having the largest body size, those in the Lower Keys being mid-sized, and those in the Upper Keys being the smallest. As such, overall the anemones attained their largest body size in the Middle Keys during several of the examined periods.

Analysis of variation in body size among the periods for all regions combined, indicated significantly larger anemones during Sep 2017 than during either Feb 2019 or Sep 2019, but not during Sept 2018 (Table 2.1B); no other time periods differed significantly. When variation among periods was analyzed for each region separately, in the Lower Keys the anemones were significantly smaller during Feb 2019 than during either Sep 2017, Sep 2018, or Sep 2019, and also smaller in Sep 2019 than Sep 2018. Time did not have a significant effect on body size in the Middle Keys, but did in the Upper Keys, where body size during Sep 2017 was significantly

larger than in Sep 2018, Feb 2019, or Sep 2019. In the Upper Keys, body size was also larger during Sep 2018 than Feb 2019, but did not differ significantly between Sep 2018 and either Feb 2019 or Sep 2019. As such, temporal analysis of body size changes did not reveal strong overall trends with season, but there was some indication that anemones were larger in Sep than Feb each year, and also were larger earlier than later during the 2-year study.

Zooxanthellae abundance within the anemone tentacles varied significantly with all 3 environmental factors examined (temperature, period, and region; Fig. 2.4A, Table 2.1C). When variation was analyzed among regions for all the periods combined, zooxanthellae abundance was significantly lower in the Lower than Middle or Upper Keys, and did not differ between the latter 2 regions. When variation was examined for each period separately, zooxanthellae abundance differed between the regions only at the beginning of the study in Sep 2017, when it was significantly lower in both the Lower and Middle Keys (which did not differ from each other) than in the Upper Keys. In terms of temporal variation among all 3 regions grouped, the zooxanthellae abundance was significantly higher during Feb 2019 than during either Sep 2017, Sep 2018, or Sep 2019, which did not differ from each other. Examination of temporal variation for each region separately revealed that significant differences in zooxanthellae abundance among periods only in the Upper Keys, where the pattern was the same as for all regions combined: it was significantly higher in Feb 2019 than during Sep 2017, Sep 2018, or Sep 2019 which did not differ from each other. Zooxanthellae abundance did not vary with time in the other 2 regions examined. Therefore, overall the abundance of zooxanthellae in anemone tentacles was higher in the Upper than Lower Keys, and higher in Feb than Sep; it was consequently lowest at the southernmost sites during the summer each year.

Chlorophyll-a concentration per cell also varied significantly with temperature, period, and region (Fig. 2.4B, Table 2.1D). For all periods combined, chlorophyll-a concentration was significantly higher in the Upper Keys than in the Lower and Middle Keys, which did not differ from each other. At the beginning of the study in Sep 2017, the chlorophyll-a concentration did not vary among regions, but a year later in Sept 2018 it was lower in the Lower Keys than in the Middle and Upper Keys, which did not differ from each other. Likewise in Feb 2019, anemones in the Upper Keys contained higher chlorophyll-a concentration than in the Middle Keys, with the Lower Keys having mid-range concentrations that did not differ from either the Upper or Middle Keys. Finally during the last sample period in Sept 2019, chlorophyll-a concentration was again lower in the Lower Keys than in the Middle and Upper Keys, which did not differ from each other. As such, the chlorophyll-a concentration per cell varied similarly to that of zooxanthellae abundance, in that it generally was higher at northern than southern sites, and during winter than summer.

In contrast to clear patterns in the above 2 microalgal characteristics, mitotic index did not vary significantly with any of the 3 environmental factors examined (temperature, period, and region; Fig. 2.4C, Table 2.1E). Overall, some aspects of the first hypothesis were supported, in that anemone abundance varied with ambient seawater temperature temporally among sample periods, but not spatially among regions, while anemone body size, zooxanthellae abundance, and chlorophyll-a concentrations all varied with ambient seawater temperature, both spatially among regions and temporally among periods. In contrast, mitotic index of did not vary with ambient seawater temperature, either spatially among regions or temporally among seasons.

Laboratory experiments

The survival rate of sea anemones varied significantly with temperature during the 6-week experimental treatments (Pearson's chi-squared test, $X^2 = 1001.9$; $p < 0.001$). Anemones exposed to the 5 most moderate temperatures (18-32°C) all experienced > 75% survival after 6 weeks (range = 78.5% at 32°C, to 93.3% at both 22 and 28°C), at rates that did not differ significantly from each other (post-hoc pairwise nominal independence tests; $p = 0.96 - 0.99$; Fig. 2.5A). In contrast, at the lower temperature of 16°C, only half the individuals (50%) survived by the end of 6 weeks, and only 30% after 10 weeks, because individuals exposed initially to 16°C continued to die even after returned to 25°C during the 4-week recovery period. At the 2 most extreme temperatures, all individuals died rapidly, within 4 weeks at the lowest temperature (14°C), and within 3 weeks at the highest temperature (34°C; Fig. 2.5A). Survival rate did not differ significantly between these 2 extreme lethal temperatures ($p = 1.00$), and was significantly lower than at all 5 other temperatures (post-hoc comparisons; $p < 0.001$ for all; Fig. 2.5A).

The initial body sizes of anemones ranged widely, from 40.37 ± 7.42 cm^2 TCSA (N = 10 anemones in the 14°C treatment group) to 162.88 ± 21.53 TCSA (N = 14 anemones in the 32°C group). Therefore, the initial body sizes of anemones varied significantly among the 8 temperature treatment groups ($p < 0.001$; $F = 6.21$), because the anemones were obtained from the field at different times during the 2 years of laboratory experiments, and they naturally fluctuated in size among seasons (Fig. 2.5B). As such, body size changes were assessed in terms of percent change from initial size at the beginning of treatments (week 0), or from the beginning of recovery (week 6), for all anemones that remained alive. In terms of percent change in body size, the anemones exposed to the moderate temperature treatments (22 and 25°C) were the only ones that grew, at substantial rates, of 33.37 ± 31.32% and 37.57 ± 26.40% (N = 17 and 15

respectively; not significantly different from each other at 6 weeks; $p = 0.99$; Fig. 2.5B). Anemones in all of the 6 other temperature treatments shrank, at rates of percent change that differed significantly from those at 22 and 25°C (and in some cases different from each other; Fig. 2.5B). The anemones at 28°C experienced slow shrinkage of only 10.90 ± 24.44% (N= 14) after 6 weeks, while those at more extreme temperatures (16, 18, and 32°C) lost most of their body size, shrinking by 98.77 ± 0.42%, 72.31 ± 6.75%, and 76.31 ± 5.50% (N = 5, 17, and 12, respectively). However, the surviving anemones in the latter 3 groups then rapidly regained body size when returned to 25°C during the 4-week recovery period, at size increase rates of 400 ± 1.34%, 295.20 ± 26.05%, and 167.08 ± 12.15% (N = 3, 15, and 11, respectively; significantly more rapid recovery of body size than for all 5 other groups, and different from each other; Fig. 2.5B). As a result, the anemones exposed to the 18 and 32°C treatments eventually regained much of their initial body size (9.44% increase in body size from week 0 to 10 for 18°C; 36.72% decrease for 32°C). In contrast, the anemones exposed to the lower temperature of 16°C remained small after 10 weeks, exhibiting a 93.88% decrease from initial body size. Anemones that had been subjected to the most moderate temperatures (22, 25, and 28°C) did not much alter their body sizes during the 4-week recovery period (only 13-19% change in body size from week 6 to week 10; not significantly different from each other; Fig. 2.5B). As such, anemones in the 3 groups that had been exposed to moderate temperatures grew during the treatment period, and then remained stable during the recovery period.

Patterns of zooxanthellae abundance within the anemone tentacles followed the same trends overall as changes in anemone body size (Fig. 2.6A). After 6 weeks at the treatment temperatures, the percent change in zooxanthellae abundance per gram wet tissue mass varied significantly among treatments ($p < 0.001$), with a significant interaction effect between time and

temperature ($p < 0.001$). Anemones exposed to the 3 moderate temperatures (22, 25, 28°C) maintained stable abundances of zooxanthellae, at rates of change that did not differ from each other but were significantly slower than for anemones at the 2 lethal (14 and 34°C) and 3 sub-lethal (16, 18, 32°C) temperatures which also did not differ from each other (Fig. 2.6A). Consequently, anemones at all of the 5 latter extreme temperatures lost most of their zooxanthellae and became nearly to completely colorless (bleached), in contrast to those at all 3 moderate temperatures which retained abundant zooxanthellae and appeared dark brown (Fig. 2.7). The surviving anemones in the 3 sub-lethal groups (16, 18 and 32°C) then rapidly regrew their zooxanthellae populations during the 4-week recovery period when they were returned to optimal temperature, at rates of 150-400% increase which differed significantly from each other (Fig. 2.7A), with anemones that had experienced 16°C not recovering completely to their pre-stress zooxanthellae abundances.

The anemone responses in terms of chlorophyll-a concentration per zooxanthellae cell deviated somewhat from those for survival, growth, and zooxanthellae abundance. Individuals exposed to moderate temperatures maintained their initial chlorophyll-a levels, but the few zooxanthellae that remained within anemones at sublethal temperatures (i.e., partially bleached anemones) exhibited a rapid increase of up to 1100% in chlorophyll concentration (Fig. 2.6B), which became significantly higher at 18 and 32°C than for all other treatments, and not different from each other ($p = 0.58$). During recovery, there was little percent change in chlorophyll-a concentration, and it did not vary significantly among the 6 groups ($p = 0.86$). The rapidly recolonizing algal cells in the anemones exposed to 18 and 32°C retained high chlorophyll-a concentrations during recovery, such that their percent change for the entire 10-week period

remained significantly higher than for the other 4 groups, and again not different from each other ($p = 0.165$, Fig. 2.6B).

Finally, mitotic index (MI) remained stable only at the 3 moderate temperatures (22, 25, 28°C; Fig. 2.6C), which did not differ significantly from each other in their percent change in MI after 6 weeks (Fig. 2.6C). In contrast, all 5 other treatments experienced rapid decrease in MI, which did not differ significantly from each other in percent change, but overlapped somewhat with that at the more moderate temperatures. During recovery, the anemones that had been exposed to sub-lethal temperatures (16 and 32°C) then exhibited high percent change in MI, as their zooxanthellae rapidly regained abundance, such that percent change in MI for the 16 and 32°C groups became significantly higher than for all other groups during recovery, and also differed between them ($p < 0.001$). Even after this temporary recovery of MI in some anemones, the percent change in MI over the entire 10-week period did not differ significantly among most groups, and indicated an overall decrease in MI for most anemones (Fig. 2.6C). Overall, results of the laboratory experiments generally supported the second hypothesis, in that most of the anemone and zooxanthellae characteristics exhibited clear peaks at moderate seawater temperatures, with reduced values indicating sublethal to lethal effects at both the high and low temperature extremes.

Discussion

General comments

We demonstrate here that the traits of zooxanthellae within corkscrew sea anemones *Bartholomea annulata* vary significantly with surface seawater temperature in the Florida Keys,

both spatially among regions and temporally among seasons. The abundance and chlorophyll-a levels of their zooxanthellae decrease during seasons and in regions with relatively high temperature, causing these anemones to partially bleach during summer in the Lower Keys. We also show experimentally that optimal seawater temperature for these anemones is ~ 22 - 25°C, with anemones exhibiting signs of stress at both cooler (18°C and lower) and warmer temperatures (28°C and higher) in terms of reduced survival and body size, as well as bleaching of their zooxanthellae in laboratory populations. Lethal temperatures for this species appear to be < 14°C and > 34°C, with the upper lethal limit approached during recent summers in the Florida Keys, and likely to be exceeded in the near future based on climate change projections (Glenn et al. 2015). Similarly, the lower lethal limit, while not reached in the Keys over the past 5 years, was exceeded in 2010 during an extreme cold event that killed many Florida reef organisms (Kemp et al. 2016) probably also including these anemones. Optimal seawater temperatures for corkscrew anemones in the Keys therefore occur during early winter to late spring (November to March or April), when temperatures range ~ 22 - 25°C. In contrast, higher temperatures during most of the rest of the year (26 - 34°C) cause thermal stress in this species.

Field observations

The 100-year pattern of air temperature variation at Key West indicates a trend of significantly increasing temperature over the past century, as well as more frequent extreme events during recent years, in terms of both high and low temperatures. The past decade includes both the coldest year and the 3 hottest years in recorded history at Key West; this trend, if not reversed, bodes ill for corkscrew anemones and many other tropical reef organisms which already live near their upper thermal limits (Muller-Parker and Davy 2001). More recent trends

over the past 2-5 years show strong seasonal variation in sea surface temperatures which regularly reach sub-lethal to lethal limits for these anemones, and are expected to become more extreme in the near future not only here but on coral reefs worldwide (van Hooidonk et al. 2016).

The correlations revealed here between seawater temperature and sea anemone traits such as abundance, body size, zooxanthellae abundance, and chlorophyll-a levels indicate that temperature likely plays an important role in the dynamic nature of this species throughout its geographical range. Mitotic index was highly variable, and this variation made any correlations with seawater temperature difficult, during both the field and laboratory studies. Mitotic index can change rapidly in cnidarians over the course of a single day (Wilkerson et al. 1988; Roopin and Chadwick 2009), making it challenging to discern large scale patterns across sites or sample periods. During 2017-2019, body parameters of *B. annulata* in the Florida Keys varied widely among regions and also among seasons within each region, but were within the range of abundances and body sizes reported for these anemones previously in both the Keys (O'Reilly and Chadwick 2017) and the US Virgin Islands (O'Reilly et al. 2018). Individuals of this species have short lifespans, high mortality, and high turnover rates relative to those of all other sea anemones examined to date, some of which can live for decades (O'Reilly et al. 2018). Their short lifespans of only ~1-2 years, and their weedy life history strategy compared to other anemones may explain in part the wide variation in traits recorded here among seasons and sites. Growth rates in particular are slower in the field than in laboratory settings, likely because field populations are exposed to more stressors such as partial predation, and suboptimal levels of light and food availability which vary seasonally (O'Reilly et al. 2018).

Seawater temperatures observed here in the Florida Keys are more seasonally variable than in most of the Caribbean, where they remain relatively more stable year-round (Briones-

Fourzan et al. 2012). Corkscrew anemones in Florida therefore must cope with wide seasonal shifts in temperature near the limit of their geographical range, which is at the northern end of the Florida Atlantic coast. However, given the increasingly variable and overall rising sea surface temperatures throughout their range, populations of *B. annulata* are expected to decline Caribbean-wide, as these anemones continue to exceed both their upper and lower thermal limits. The patterns observed here in traits of field populations also likely varied due to the contributions of other environmental factors such as dissolved nutrient and plankton levels, light intensity, sedimentation rate, and precipitation runoff causing salinity variation, some of which may co-vary seasonally and spatially with temperature. Especially, levels of solar irradiance co-vary with temperature on a seasonal basis, and both microalgal abundance and chlorophyll-a concentrations in reef anemones are known to decrease when irradiance is high (such as during summer vs. winter, or in shallow vs. deep habitats), as a mechanism of physiological acclimation to capture fewer photons when light becomes saturating (Dixon et al. 2014).

The natural experiment created by Hurricane Irma which passed over our study sites allowed us to quantify rates of recovery by these anemones after a major storm. Three of the original 6 field sites exhibited no recovery during the year following the hurricane, indicating that some populations are highly vulnerable and could become locally extinct if storm disturbance becomes more frequent, which is also predicted by climate change modeling (Knutson et al. 2013). While abundances immediately before the hurricane were not quantified, comparison with historical data allowed assessment of population recovery rates at the 3 sites that retained anemones. Recovery from acute disturbance potentially may be rapid, in that anemone abundance ~1 year post-hurricane (2018; present study) can return to and even exceed that recorded beforehand (O'Reilly and Chadwick 2017). The abundances of *B. annulata*

recorded here (1.2-6.8 individuals m^{-2}) were much higher than those reported for this species in the Yucatan, (~ 0.01 individuals m^{-2} = ~ 23 individuals 0.25 ha^{-1}; Briones-Fourzan et al. 2012) and the US Virgin Islands (0.05 - 0.44 individuals m^{-2}; O'Reilly et al. 2018). However, our measurements focused on small areas of maximal anemone presence within each site, so may have overestimated their abundance over larger reef areas. Mechanisms of potential recovery from disturbance by this species include rapid recruitment and population turnover (O'Reilly et al. 2018), likely due to the frequent arrival of sexually-produced larvae from other populations, which then metamorphose and aggregate to form small, genetically diverse groups of polyps (Titus et al. 2017). Rapid individual growth rates also allow these anemones to quickly reach adult size (Jennison 1981, O'Reilly and Chadwick 2017), although the maximal body size observed here in Florida (~ 207 cm^2 TCSA) was smaller than those recorded in the Yucatan (380 cm^2; Briones-Fourzan et al. 2012) and Virgin Islands (452 cm^2; O'Reilly et al. 2018).

Laboratory experiments

Responses of *B. annulata* anemones to high temperature were similar to those of some temperate sea anemones, which also decrease their body sizes under heat stress, including *Anthopleura elegantissima* (Sebens 1980), *Actinia equina* (Chomsky et al. 2004), and *Diadumene lineata* (Ryan 2018). Temperate anemones often exhibit a wide range of thermotolerance; *Metridium senile* can survive at temperatures ranging < 0-27°C, although prolonged exposure to high temperature reduces their rate of clonal reproduction (Glon et al. 2018). However, in contrast to our observation that temperature variation caused significant change in microalgal abundance within the anemone tentacles, the microalgal abundances in *A. elegantissima* anemones did not vary among temperature treatments ranging 6-24°C, nor did

mitotic index or the chlorophyll-a content of algal cells (Verde and McCloskey 2001). This difference in response may be due in part to tropical anemones hosting more thermally-sensitive algal populations, which are closer to their upper thermal limits than are those in temperate regions (Muller-Parker and Davy 2001). Overall, thermal ranges for tropical species are narrow compared to temperate species, due to the relatively stable and less seasonal nature of the tropics (Woolsey et al. 2015). However, here we demonstrate a remarkably wide range of temperature tolerance of ~ 20°C (14 – 34°C) for a tropical species. Levels of genetic variation in *B. annulata* are known to differ significantly among areas of the Caribbean (Titus et al. 2017), so the thermotolerance levels reported here may differ from those of populations further south. Differential, heritable thermotolerance occurs among populations of scleractinian corals (Dixon et al. 2015), and reef cnidarians which live at low latitudes and thus more stable temperature regimes may be more susceptible to stress from climate change-induced warming than their higher latitude counterparts (Woolsey et al. 2015).

The observed intolerance of this species to prolonged high temperature could result in future range shifts. Tropical corals in Japan recently have expanded their range poleward by 14 km per year, indicating that some temperate zones may act as sanctuaries for tropical species, as sea surface temperature rises beyond their physiological limits near the equator (Yamano et al. 2011). In the Indo-Pacific, increasing temperatures are inducing the "tropicalization" of temperate reefs, with species such as *Entacmaea quadricolor* and *Heteractis crispa* extending their geographic ranges southward (Malcom and Scott 2017). *Stichodactyla haddoni* also has significantly expanded its range south toward warming temperate waters, along with its associated commensal crustaceans (Scott et al. 2015). Conversely, high temperature stress near the equator induces bleaching in giant anemones *H. magnifica* in Moorea, with negative cascade

effects on associated anemonefishes (Beldade et al. 2017). If warming seas drive individuals of *B. annulata* farther poleward, this could result in competition with other types of benthic space-occupiers north along the Atlantic coast of the United States, northwest into the Gulf of Mexico, and southward along the coast of Brazil, creating new interactions with unknown ecological consequences. It is also unknown whether associated cleaner shrimp could keep pace with range shifts in these host anemones, as the shrimp may self-recruit mostly to home reefs (Gilpin and Chadwick 2017). Given the dependence of Caribbean coral reef fish health on the presence of cleaner shrimp that form obligate associations with this sea anemone (reviewed in O'Reilly et al. 2018; Huebner et al. 2019), the approach of upper thermal limits for corkscrew anemones throughout much of their geographic range is cause for conservation concern.

Conclusions

The present study demonstrated the effects of thermal stress on an ecologically important sea anemone, in that outside of the optimal temperature range of 22-25°C detrimental effects on growth and survival occur including loss of body size and symbiotic zooxanthellae. Bleaching in this species was shown to be reversible if temperatures return to optimal range, allowing zooxanthellae populations to recover within anemone tissues. Current temperature trends in the Florida Keys indicate that *B. annulata* populations are living near the upper thermal limit for this species, and results from this study indicate the potential effects of long term temperature stress. As an ecologically important species that supports coral reef fish health and diversity (Grutter et al. 2003), conservation management in the Florida Keys should focus on monitoring current

populations, and limiting commercial harvest during periods of thermal stress as well as reproductive seasons (O'Reilly and Chadwick 2017).

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Figures and Tables

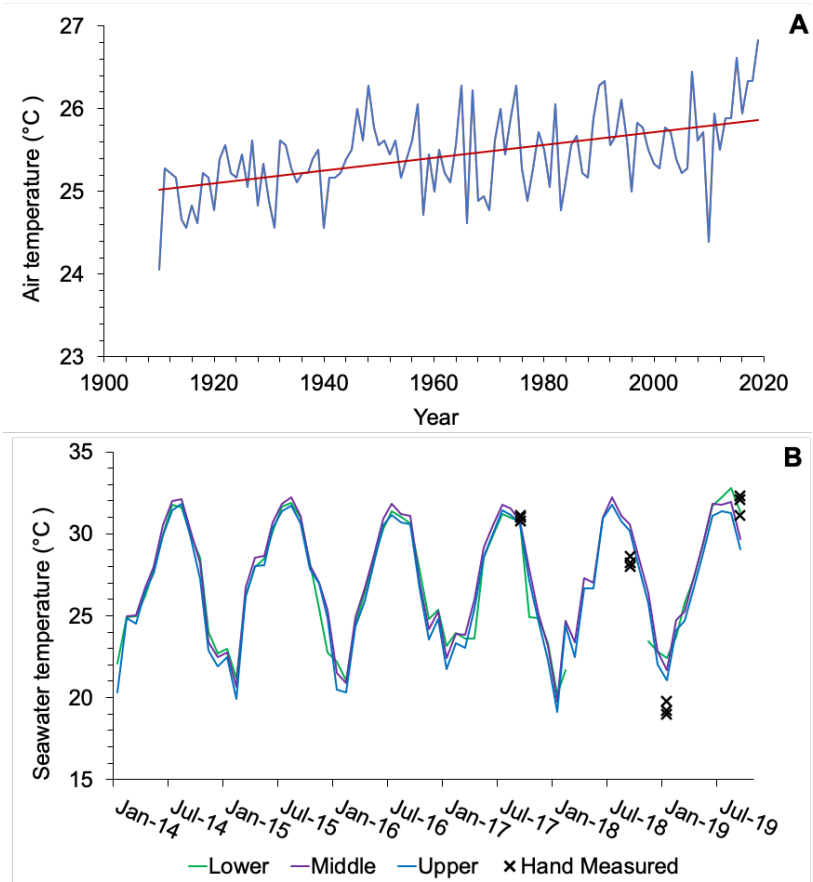

Fig. 2.1. Patterns of short- and long-term variation in temperature in the Florida Keys, USA, A: Annual mean air temperature over the last century (1910-2019) at Key West (Lower Florida Keys), calculated from NOAA Regional Climate Centers (http://xmacisrcc-acisorg/), as recorded daily at station Key West (Station ID 12858 WBAN), B: Monthly mean surface seawater temperature over 6 years (2014-2019) in 3 regions of the Keys: Upper (blue), Middle (purple) and Lower Keys (green), calculated from the NOAA National Data Buoy Center (https://wwwndbcnoaagov/). Temperature data from a nearshore buoy adjacent to field sites within each region were used: Lower Keys (KYWF1 24°33'21" N 81°48'28" W), Middle Keys (VCAF1 24°42'40" N 81°6'24" W) and Upper Keys (BNKF1 25°5'12" N 80°31'8" W; see also Fig. 2.2). Temperatures measured by hand at each study site during field sampling periods for sea anemone characteristics in the present study (2017-2019) also are shown as symbols (x's). The monthly data indicate that in all 3 regions, the annual temperature maximum occurs in July-August, and the annual minimum in January-February.

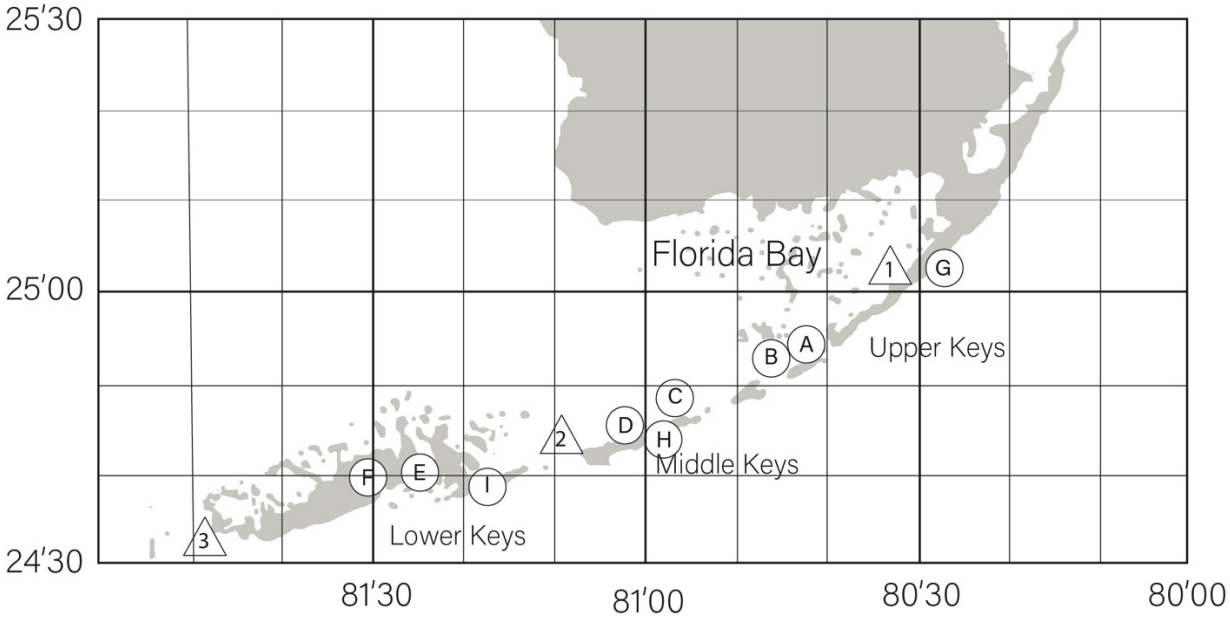

Fig 2.2. Map of the Florida Keys, USA, showing the locations of 6 field sites examined prior to the present study (shown as letters A-F inside circles), and 3 additional sites examined during the present study (shown as letters G-I inside circles), for characteristics of corkscrew sea anemones *Bartholomea annulata*. Also shown are the locations of 3 NOAA weather buoys (shown as numbers inside triangles) used to calculate seawater temperature during the present study within each of 3 regions containing the field sites (Upper, Middle, and Lower Keys; modified after O'Reilly and Chadwick 2017). All field sites were located in nearshore areas along the Florida Bay side of the Keys. Previously-examined sites: (A) Indian Channel, (B) Robbies, (C) Quarry,

(D) Tiki Hut, (E) Bowman's Channel, and (F) Cudjoe. Additional sites examined during the present study: (G) Buttonwood Bay, (H) Fire Academy, and (I) Horseshoe Quarry. The 3 NOAA weather buoys (http://www.ndbc.noaa.gov/) were: (1) Upper Keys buoy (Buoy BNKF1; 25°5'12" N, 80°31'8"; note that this buoy differed in location from that used in O'Reilly and Chadwick 2017, due to the location of the additional Upper Keys site G), (2) Middle Keys buoy (Buoy VCAF1; 24°42'40" N, 81°6'24" W), and (3) Lower Keys buoy (Buoy KYWF1; 24°33'21" N, 81°48'28" W). Map created by C Barker.

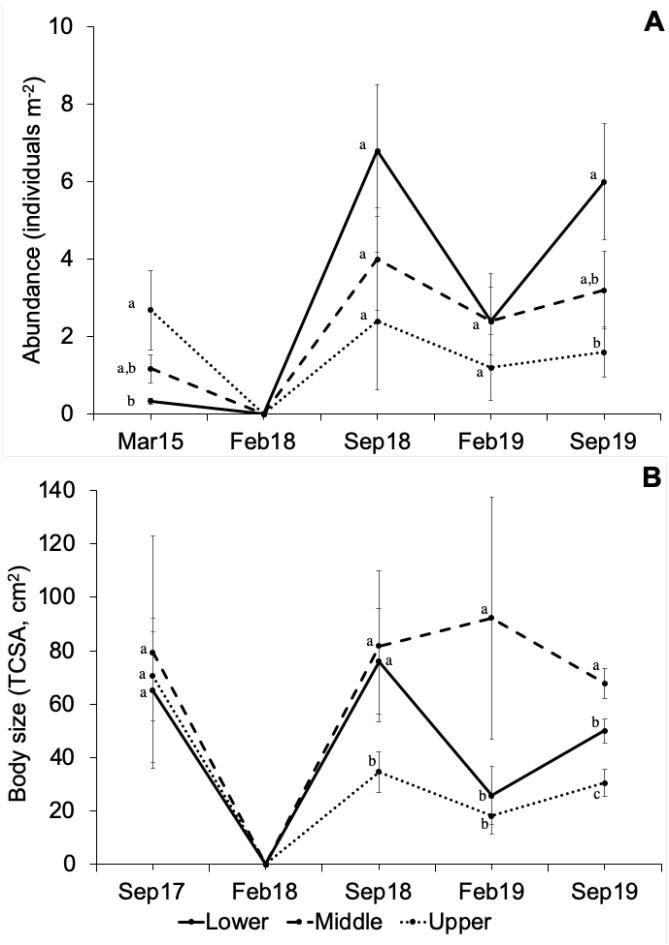

Fig 2.3. Variation in characteristics of corkscrew sea anemones *Bartholomea annulata* over 2 years among 3 regions of the Florida Keys, USA (Lower Keys [solid black line], Middle Keys [dashed line], and Upper Keys [dotted line]), A: Anemone abundance (number of individuals m^{-2}), B: Anemone body size (tentacle crown surface area [TCSA] in cm^2). For Sep 2017, N = 10 anemones per site per sample period x 2 sites per region = 20 anemones per region. Then Hurricane Irma in Sep 17 caused all anemone populations to drop too low to sample in Feb 2018. For Sep 2018 – Sep 2019, N = 10 anemones per site per sample period x only 1 site remaining

per region = 10 anemones per region, because the other site in each region never recovered anemones. Note that because no data were collected on anemone abundance in Sep 2017, data from Mar 2015 at these sites were used as a pre-hurricane reference point (after O'Reilly and Chadwick 2017). Regions that share a lower case letter did not differ significantly during each period. Data are presented as means ± 1 SE; see Table 1 and text for details.

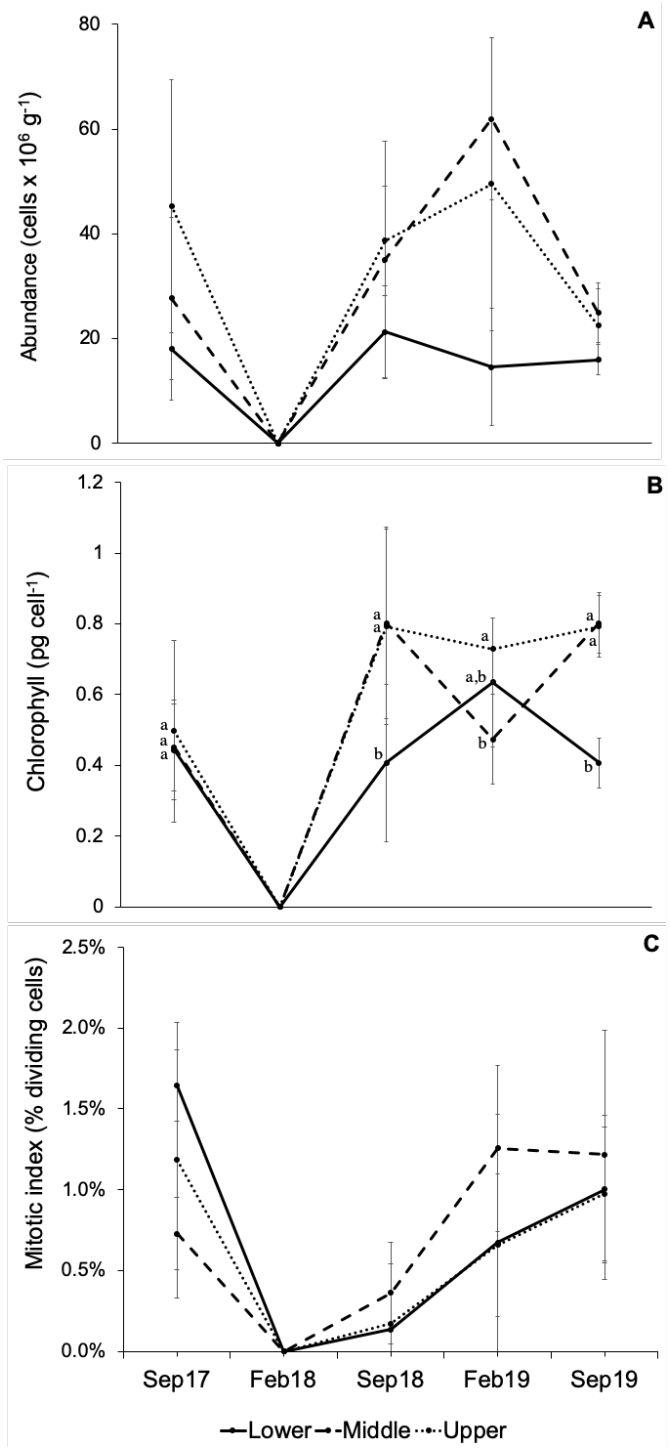

Fig 2.4. Variation in characteristics of microalgal cells in the tentacles of corkscrew sea anemones *Bartholomea annulata* over 2 years among 3 regions of the Florida Keys, USA (Lower Keys [solid black line], Middle Keys [dashed line], and Upper Keys [dotted line]), A: Abundance of microalgal cells (number of cells per gram tentacle wet mass), B: Concentration of chlorophyll a per microalgal cell, C: Mitotic index of microalgal cells (percent of cells dividing).

In B, regions that share a lower case letter did not differ significantly during each period; no letters are indicated in A and C, because there were no significant differences between any of the regions. For analysis details, see Fig. 2.3. Data are presented as means ± 1 SE.

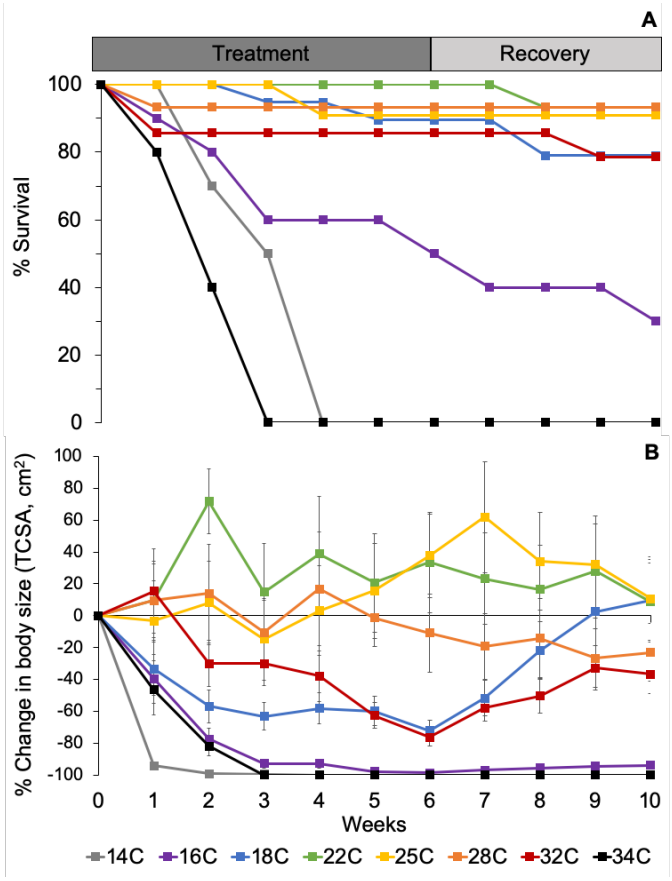

Fig 2.5. Variation in characteristics of corkscrew sea anemones *Bartholomea annulata* with experimental temperature treatments under laboratory conditions; shown are percent changes in, A: Survival of individuals, B: Body size of individuals (tentacle crown surface area [TCSA] in cm^2). N = 10 - 19 individuals per treatment. Treatments were applied for 6 weeks, then all groups were returned to the moderate culture temperature of 25°C for 4 weeks, to determine rates of recovery after treatments were removed. Data are presented as means ± 1 SE.

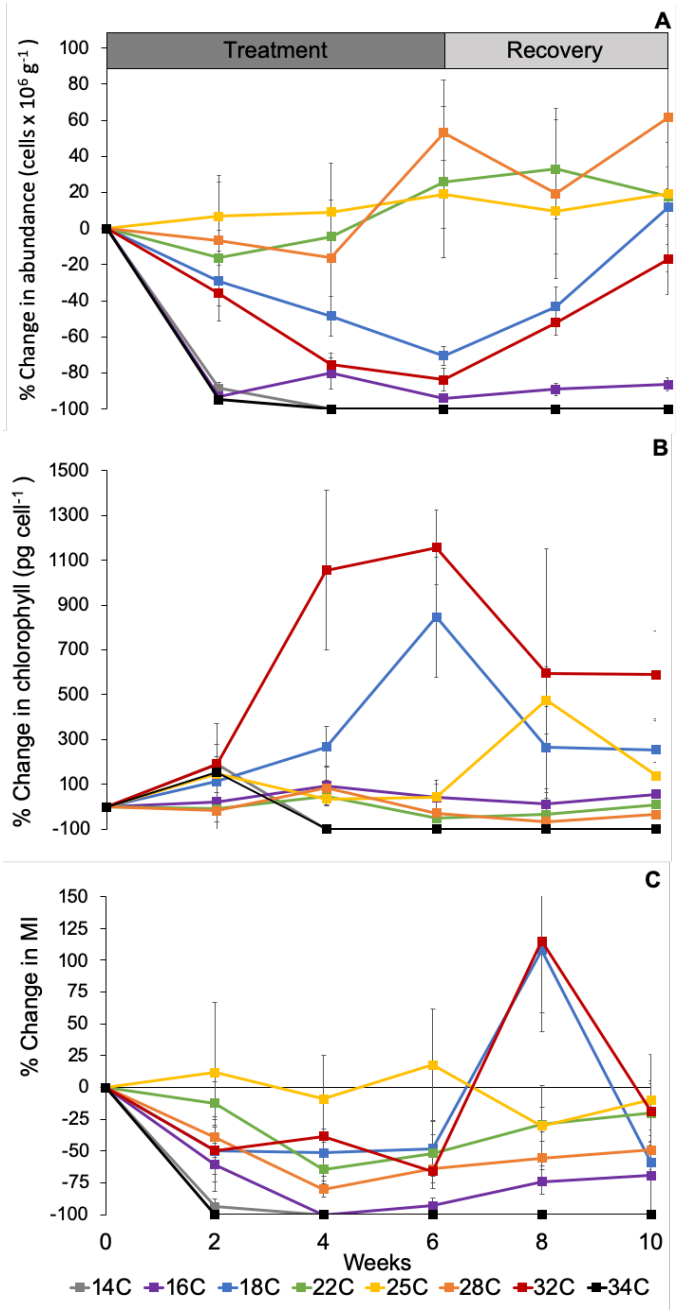

Fig 2.6. Variation in characteristics of endosymbiotic microalgae inside the tentacles of corkscrew sea anemones *Bartholomea annulata* with temperature treatments under laboratory conditions; shown are percent changes in, A: Abundance of microalgal cells per wet mass of anemone tentacle, B: Concentration of chlorophyll a per microalgal cell, C: Mitotic index of microalgal cells (percent of cells dividing). N = 10 anemones per treatment, with 1 tentacle sampled per anemone every 2 weeks. Treatments were applied for 6 weeks, then all groups were returned to the moderate culture temperature of 25°C for 4 weeks, to determine rates of recovery after treatments were removed. Data are presented as means ± 1 SE.

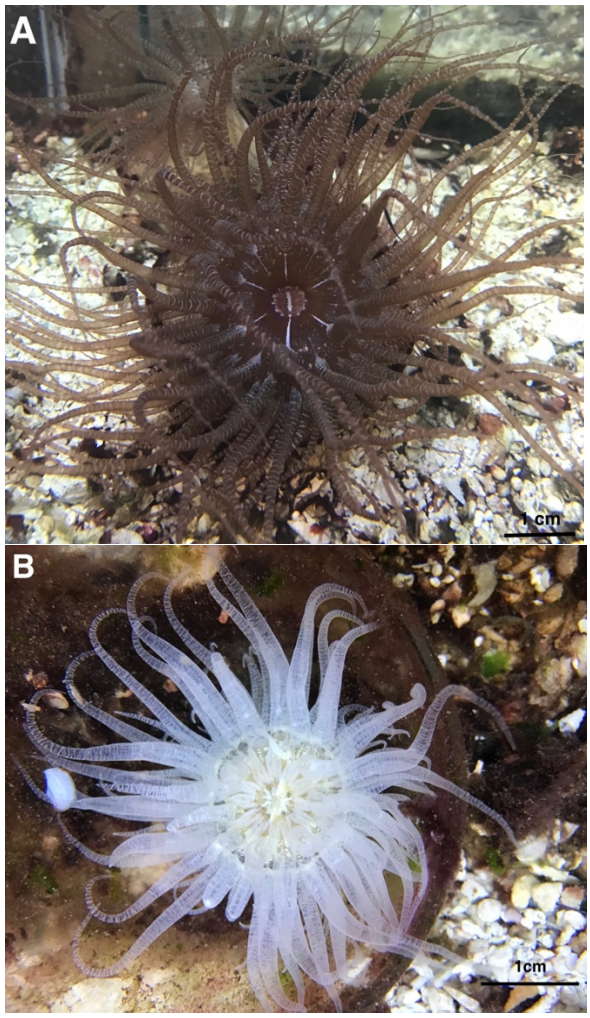

Fig 2.7. Photographs of corkscrew sea anemones *Bartholomea annulata* in experimental laboratory treatments, A: Pre-treatment individual in normal culture conditions, showing dark brown color indicating abundant microalgal cells inside the anemone tentacles, B: Individual after 6 weeks in the 32°C temperature treatment, showing pale transparent tissues that indicate bleaching or loss of nearly all microalgal cells. Note in both individuals the slit-like mouth at center, the surrounding oral disk with radial striations of anemone animal pigment, and the long thin tentacles with corkscrew-like spiral nematocyst batteries. See Fig 2.6A for microalgal abundances in each treatment.

Table 2.1. Results of ANOVAs followed by Tukey post-hoc pairwise comparisons tests for variation in the characteristics of sea anemones *Bartholomea annulata* and their endosymbiotic microalgae, with temperature, region, and period of examination at field sites in the Florida Keys, USA. Shown are results for 3 regions (Upper, Middle, and Lower Keys) each examined during 4 periods (Mar 2015 or Sep 2017 depending on the characteristic, Sep 2018, Feb 2019, Sep 2019) that varied in surface seawater temperature; A: sea anemone abundance, B: sea anemone body size, C: microalgal cell abundance, D: chlorophyll concentration per microalgal cell, E: mitotic index of microalgal cells. See text for details

A. Sea anemone abundance

Three factor ANOVA	Factor	F-statistic	P value
	Temp+Region+Period	4.434	<0.001*
	Temperature	16.080	<0.001*
	Region	1.635	0.200
	Period	5.845	<0.001*

All Regions: Pairwise comparisons between periods

	Mar 15	Sep 18	Feb 19	Sep 19
Mar 15		0.002*	0.937	0.145
Sep 18			0.006*	0.351
Feb 19				0.350
Sep 19				

Separate Regions:
Lower Keys F=5.358, p=0.004*

	Mar 15	Sep 18	Feb 19	Sep 19
Mar 15		0.008*	0.697	0.024*
Sep 18			0.079	0.968
Feb 19				0.193
Sep 19				

Middle Keys: F=1.122, p=0.354
Upper Keys: F=1.989, p=0.134

B. Sea anemone body size

Three factor ANOVA	Factor	F-statistic	P value
	Temp+Region+Period	12.200	<0.001*
	Temperature	13.590	<0.001*
	Region	13.100	<0.001*
	Period	6.182	<0.001*

All Periods: Pairwise comparison between regions

	Lower	Middle	Upper
Lower		<0.001*	0.112
Middle			<0.001*
Upper			

Separate Periods:
Sep 17: F=1.053, p=0.355
Sep 18: F=15.910, p= <0.001*

	Lower	Middle	Upper
Lower		0.81	<0.001*
Middle			<0.001*
Upper			

Feb 19: F=22.62, p<0.001*

	Lower	Middle	Upper
Lower		<0.001*	0.800
Middle			<0.001*
Upper			

Sep 19

	Lower	Middle	Upper
Lower		0.039	0.007
Middle			<0.001*
Upper			

All Regions: Pairwise comparisons between periods

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		0.718	0.002*	0.007*
Sep 18			0.113	0.233
Feb 19				0.984
Sep 19				

Separate Regions:
Lower Keys: F=11.31, p<0.001*

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		0.545	<0.001*	0.265
Sep 18			<0.001*	0.040*
Feb 19				0.063
Sep 19				

Middle Keys: F= 0.718, p=0.547
Upper Keys: F=42.15, p<0.001*

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		<0.001*	<0.001*	<0.001*
Sep 18			0.051	0.643
Feb 19				0.460
Sep 19				

C. Microalgal abundance

Three factor ANOVA	Factor	F-statistic	P value
	Temp+Region+Period	5.775	<0.001*
	Temperature	10.3	0.002*
	Region	6.285	0.002*
	Period	4.495	0.004*

All Periods: Pairwise comparison between regions

	Lower	Middle	Upper
Lower		0.028*	0.002*
Middle			0.703
Upper			

Separate Periods:
Sep 17: F=10.060, p<0.001*

	Lower	Middle	Upper
Lower		0.263	<0.001
Middle			0.016*
Upper			

Sep 18: F=1.673, p=0.207
Feb 19: F=2.199, p=0.130
Sep 19: F=0.724, p=0.494

All Regions: Pairwise comparisons between periods

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		0.999	0.017*	0.766
Sep 18			0.069	0.771
Feb 19				0.004*
Sep 19				

Separate Regions:
Lower Keys: F=0.840, p=0.479
Middle Keys: F=2.068, p=0.118
Upper Keys: F=5.671, p=0.002

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		0.949	0.039*	0.265
Sep 18			0.031*	0.671
Feb 19				0.001*
Sep 19				

D. Chlorophyll

Three factor ANOVA	Factor	F-statistic	P value
	Temp+Region+Period	5.832	<0.001*
	Temperature	4.974	0.027
	Region	6.113	0.003
	Period	6.515	<0.001*

All Periods: Pairwise comparison between regions

	Lower	Middle	Upper
Lower		0.886	0.004*
Middle			0.017*
Upper			

Separate Periods:
Sep 17: F=0.346, p=0.709
Sep 18: F=7.689, p=0.002*

	Lower	Middle	Upper
Lower		0.005*	0.007*
Middle			0.995
Upper			

Feb 19: F=4.826, p=0.016*

	Lower	Middle	Upper
Lower		0.149	0.498
Middle			0.013*
Upper			

Sep 19: F=9.634, p<0.001*

	Lower	Middle	Upper
Lower		0.176	0.044*
Middle			<0.001*
Upper			

All Regions: Pairwise comparisons between periods

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		<0.001*	0.027*	0.976
Sep 18			0.806	0.017*
Feb 19				0.159
Sep 19				

Separate Regions:
Lower Keys: F= 2.531, p=0.069
Middle Keys: F=16.250, p<0.001

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		<0.001*	0.983	0.218
Sep 18			<0.001	<0.001
Feb 19				0.199
Sep 19				

Upper Keys: F=3.675, p=0.019

	Sep 17	Sep 18	Feb 19	Sep 19
Sep 17		0.023*	0.103	0.370
Sep 18			0.947	0.654

	Feb 19				0.926	
	Sep 19					

E. Mitotic index

Three factor ANOVA	Factor	F-statistic	P value
	Temp+Region+Period	2.062	0.061

Chapter 3

Effects of temperature on the balance between respiration and photosynthesis in the symbiotic sea anemone *Bartholomea annulata*

Abstract

Climate change in marine environments drives shifts in abundance and distribution of key coral reef species. The physiological response to temperature of the widely distributed and ecologically important sea anemone *Bartholomea annulata* is unknown. We determined temperature effects on the physiology of *B. annulata* in terms of changes in whole-animal respiration (resting metabolic rate, RMR), net photosynthetic rate (NPR), as well as cellular level effects to electron transport system activity (potential metabolic activity, PMA) by enzyme assays. Acclimation temperature had a significant effect on all physiological parameters in that high temperature increased RMR, decreased NPR, decreased the photosynthesis to respiration ratio (P:R), and increased PMA. As a result, anemones at high temperature were energetically compromised, and in some cases even put into a negative energy balance, thus making them potentially more vulnerable to bleaching.

Introduction

Cnidarian-algal symbioses are common from tropical to temperate ecosystems, allowing the cnidarian partner to maintain high productivity in diverse and sometimes nutrient-poor

environments (Linton and Warner 2003). In this tightly evolved partnership the animal host provides a concentrated source of nutrients, including dissolved inorganic carbon (DIC) and nitrogen (ammonia, NH_3/NH_4^+) via uptake from seawater and transfer to microalgae cells (Bedwell-Ivers et al. 2017; Cantrell et al. 2015; Roopin et al. 2011). This endosymbiotic relationship consists of the cnidarian providing inorganic nutrients such as nitrogen to the photosynthetic algae, which then in turn provide energy to the host in the form of photosynthate, and the amount varies with the degree of dependence of the host on that energy source (Muller-Parker et al. 2015) Some endosymbionts are capable of supporting up to 100% of the host's energic needs (Muller-Parker and Davy 2001). This mutualism can begin to break down if environmental conditions become stressful, with high temperatures and light levels inducing bleaching, the loss of microalgae from cnidarian tissues. The bleaching response is well known for tropical reef-building coral species (Weis 2008) and are preceded by changes to the physiology of the cnidarian, such as a disproportional increase in respiration rate compared to photosynthetic rates (Paradis et al. 2019). Equivalent quantitative studies on ecologically important coral reef cnidarians, such as soft-bodied sea anemones, are lacking.

One such ecologically important species in the Caribbean is the corkscrew sea anemone *Bartholomea annulata*, with a wide geographic range from the Gulf of Mexico to Brazil and extending as far east as Bermuda. This species hosts endosymbiotic algae belonging to Symbiodinicae clades A and C (Grajales et al. 2016), and at least 6 ectosymbiotic crustacean species (Huebner et al. 2019), including the Pederson cleaner shrimp, the major reef fish cleaner on Caribbean reefs (Huebner and Chadwick 2012; Titus et al. 2019). Despite their ecological importance and commercial popularity (O'Reilly and Chadwick 2017), little is known about the thermotolerance of this species, or what environmental conditions are optimal for their growth

and development. The relationship between temperature, energy balance, and metabolic health can be studied by a combination of whole-organism respiratory physiology and cellular physiology of the enzymatic components of energy producing metabolic pathways.

Respiratory and photosynthetic rates have been examined in sea anemone species previously, however most studies have focused on temperate species or species of low ecological importance such as *Exaiptaisa pallida* (Davy and Cook 2001; Goulet et al. 2005). The ratio of photosynthetic rate to respiratory rate, the P:R ratio, is a commonly used metric for determination of the energy budget of cnidarian holobionts as well as other photosynthetic organisms. A P:R ratio of 1 would indicate equal rates of oxygen production and consumption, with <1 implying a net energy deficit and >1 an excess of photosynthetically-derived energy (Coles and Jokiel 1977). Anemones in general have a lower P:R ratio than tropical coral species, with corals relying extensively on algal photosynthesis for energy (Fitt et al. 1982). Additionally, tropical sea anemone species have higher ratios than temperate anemones, indicating reduced dependence on microalgae for nutritional needs in temperate species (Muller-Parker and Davy 2001). P:R ratios at high irradiance are slightly lower in temperate anemones and also lower than in tropical corals (Davies, 1977, 1991; Muscatine et al., 1984). Furthermore, rapid attenuation of light intensity with depth, seasonal changes in irradiance, frequent cloud cover, shading by algae, and a relatively more abundant food supply all contribute to an even greater lowering of the P:R ratio in temperate anemones (Verde, 1993; Davy etal., 1996; Shick and Dykens 1984; Fitt et al., 1982). As a result, temperate anemones potentially depend more on heterophagy, and thus a lower P:R ratio does not have as significant negative effects on energy budgets as seen in corals.

Photosynthetic and respiratory rates vary with temperature; when temperature increases, respiration rate increases as is typical for most animals under thermal stress (Shick 1991). Geographically separated populations of the same anemone species may have significantly different respiration rates based on temperature regime (Walsh and Somero 1981). Some species have higher acclimation capability and may not vary oxygen consumption rates significantly with temperature, while other species are more susceptible to change (Shick 1991). Tropical species of sea anemones, in particular, are subject to higher temperatures year-round, leading to high metabolic cost. This may in part explain the high number of cnidarian-algal symbioses in the tropics: high abundance of zooxanthellae and resultant high P:R ratios may have been selected for as a mechanism to mitigate energetic costs (Chomsky et al. 2004).

Pre-conditioning can also play a part in acclimation to thermal stress. Short-term exposure to near-lethal temperatures may confer a degree of resistance if similar temperatures are encountered in the future (Cossins and Bowler 1987). Pre-conditioning has been shown in aquatic invertebrates to lower Q_{10} values; those animals that are more acclimated to extreme temperatures are more temperature insensitive (Vernberg and Vernberg 1969). In corals, a more stressful thermal history resulted in significantly lower respiration rates when corals were exposed to high temperature, approaching lethal limits (Castillo and Helmuth 2005). This phenomenon has also been demonstrated in anemones; pre-conditioned individuals were better able to resist bleaching (Hawkins and Warner 2017)

Additionally, interaction of increasing temperature coupled with increasing light level increases photosynthetic rate (Verde and McCloskey 2007). A rise in temperature increases compensation intensity, the point at which photosynthesis equals 0 (Goulet et al. 2005).

However, photoinhibition due to reduction in enzymatic function and damage to photosystems may occur at high temperatures (Lesser and Shick 1989).

At the cellular level, utilizing respiratory electron transport system (ETS) activity to determine the maximum rate of oxygen consumption can allow estimation of the potential metabolic activity (PMA) of marine organisms. This measure allows a comparison of the maximum possible metabolic rate with *in-vivo* whole-animal respiratory measurements. The method proposed by Packard (1971), has been used for a variety of species including phytoplankton (Packard and Taylor 1968), ctenophores and coelenterates (King and Packard 1975), and more recently crayfish (Simčič et al. 2014), and scleractinian corals (Agostini et al. 2013). Aerobic scope represents the difference between PMA, or surplus oxygen available for activities other than those necessary for basic function, such as growth, reproduction, and locomotion, and RMR. These activities are at their highest level of functioning at the temperature of the highest aerobic scope (Clark et al. 2013). The oxygen- and capacity-limited thermal tolerance theory postulates that ectotherms have evolved to maximize fitness at a given temperature range where aerobic scope is highest (Portner et al. 2017).

Thermotolerance exists as a range for an organism, with maximum and minimum temperatures for survival. The width of this zone can change with species, population, age, and phenotypic plasticity, and this in turn can influence geographic distribution (Schulte et al. 2011). Conversely, for tropical species that have lived for generations under very narrow (or tight) thermal conditions, even small increases in maximal environmental temperatures could push a species out of its tolerance zone and potentially threaten its existence (Jokiel and Coles 1990).

In this study we investigate the effect of temperature on physiological function of the ecologically important symbiotic sea anemone *Bartholomea annulata* in terms of respiration rate,

irradiance-dependent photosynthetic rate, and ETS activity in response to increasing temperature.. Understanding the physiology of this species will allow for better management decisions for populations facing climate change induced environmental stressors as well as optimal culture conditions for captive individuals.

Methods

The present laboratory study was conducted on individuals of *Bartholomea annulata* obtained from commercial suppliers (KP Aquatics, Tavernier, and Sea Critters of the Florida Keys, Key Largo, Florida, United States). Anemones were acclimated to laboratory conditions at Auburn University for 1 month prior to experimentation in 75L tanks supplied with overhanging filters (Aqueon QuietFlow20, Aqueon), tank heaters (Eheim Jager TruTemp), and T5 florescent lights (AquaticLife Marquis Marine 24" Dual Lamp T5 HO). Conditions in the culture tanks mimicked those in the coral reef habitats occupied by this anemone: 25.5 ± 1 °C; 34 ± 1 ppt salinity; 12:12 hour light regime; PAR 61.8-88.9 mmol photons m^{-2} s^{-1}; nutrient levels NH_4^+ <0.5 μmol L^{-1}, NO_2^- 0.01 μmol L^{-1}, and NO_3^{-2} 0.1 μmol L^{-1} (see Roopin and Chadwick 2009, Huebner et al. 2012, Cantrell et al. 2015, for more details of laboratory culture conditions).

Respiratory measurements

Prior to trials, anemones (N=40, weight range 0.3-3.3 g wet mass) were acclimated to the experimental temperature at a rate of 1°C per day change from maintenance temperature of 25°C to one of the 5 experimental temperatures: 18, 22, 25, 28, or 32°C (N=8 per treatment). After experimental temperature was reached and maintained for 10 days, animals were transported

from Auburn's main campus to the North Auburn Fisheries lab for respirometry. Anemones were fed once per week (thawed *Artemia* sp., Frozen Brine Shrimp, San Francisco Bay Brand) during acclimation but were starved for 48 hours prior to respiratory measurements.

Oxygen consumption (resting metabolic rate RMR) or production (net photosynthetic rate NPR) was measured using a Witrox-4 fiber optic oxygen sensing system (Loligo Systems, Denmark) in a closed, recirculating respiratory chamber and reported as MO_2. Optodes attached to the control box were inserted into a port in the recirculation tubing of each chamber to monitor the increase or decline in the partial pressure of O_2 (PO_2) during photosynthesis and respiration, respectively. The optodes use blue-green LED lights to excite dye particles in the O_2 sensor, emitting florescence when it encounters an O_2 molecule. The degree of florescence is correlated to the PO_2 of the dye, which is equal to that of the surrounding medium. An intermittent flow (stop-flow) design was used, with 8 animals (max number per run) placed into individual acrylic chambers (400 mL), each fitted with 4 ports (2 inflow, 2 outflow; 1 mm outer diameter), Tygon tubing (Pentair, Apopka, FL, USA) all with 4mm mesh coverings to prevent anemones from being pulled into the pumps. All chambers were placed in a large water bath (~300L) filled with seawater (34 ppt) at the acclimation temperature controlled by a chiller/heater unit (TECO TK-2000, TECO USA), and aerated to maintain ~100% saturation. A recirculating pump was attached to each chamber to ensure proper mixing and flow around the probe (closed pump). The flow rate was maintained at ~9.5ml/s, which was fast enough to ensure full tentacle extension and optimal gas exchange (Sczeback et al. 2012). A second attached pump flushed the chamber with water from the water bath between runs (flush pump; Eheim GmbH & Co., Deizisau, Germany). Activation of closed and flush pumps was achieved using AutoResp software (Loligo Systems, Viborg, Denmark) and a Netio Bluetooth control device. Prior to

placing anemones into chambers and at the conclusion of a trial, control runs of 30 mins each were performed to determine background bacterial respiration. Anemones were weighed to determine wet mass and placed into chambers. Once in chambers, anemones were allowed to acclimate for at least 30 minutes, with fresh water flushing through the chambers, to allow for recovery from handling stress and full extension of tentacles. Following acclimation, respiration rates were measured via intermittent respirometry consisting of multiple, sequential loops. Each experimental "loop" ran for 1 hour and 45 minutes (20 minute flush, 5 minute wait, 1 hour 20 minute measurement period). During the measurement period, water was recirculated through the chamber via a closed loop and the decline in DO (mg/L) recorded by AutoResp software. The goal during each loop was to initially flush water through a given chamber until the water within reached 100% dissolved oxygen concentration, and then declined during the subsequent measurement period to ≥ 80% saturation. This approach was designed to ensure that respiration rates were measured under normoxic conditions that anemones would typically experience in the field. Respiration trials to determine RMR (resting metabolic rate) of the anemone plus symbiotic algae consisted of 4-5 sequential loops with chambers kept in darkness (0 mmol photons m^{-2} s^{-1} PAR) to prevent photosynthesis. Respirations rates were averaged across all loops for a given animal to estimate RMR at the experimental temperature.

Photosynthetic trials to determine NPR (net photosynthetic rate; MO_2) were conducted on the same individuals (N=8) from respiration trials after a 1-2 day recovery period in holding tanks at the given treatment temperature. Light levels on Florida Keys coral reefs vary widely with depth, time of day, and season, ranging from ~10-1200 mmol photons m^{-2} s^{-1} at 3m to a maximum of only ~450 mmol photons m^{-2} s^{-1} at 18m depth (Lesser 2000). To assess how variation in irradiance contributes to changes in photosynthetic rate, trials were performed using

LED aquarium lights (Model HYA05-LENS- 55*3W-B, Galaxyhydro). Intermittent respirometry followed the same procedure described previously except that light levels were increased after each loop (12 loops total per individual) to measure RMR under an increasing light regime: 50, 100, 150, 250, 350, 450, 550, 650, 750, 850, 950, 1050 PAR, mmol photons m^{-2} s^{-1}. Light level was determined inside of a respirometry chamber without an anemone using a QSL-2001 Scalar PAR Sensor (Biospherical Instruments, San Diego, CA, USA). At the conclusion of each trial, all experimental equipment was cleaned with 5 ml bleach per gallon tap water and rinsed in DIH_2O to prevent bacterial buildup that may affect respiratory measurements. NPR was calculated using the formula [(O_2initial-O_{2final})*chamber volume]/time/g wet mass and was reported in units of mg O_2/g wet weight/hr.

ETS (Electron transport system) assays

An additional N= 18 individuals of *B. annulata* were acclimated to one of three temperatures: 18, 25, and 32°C (8 individuals per treatment; identical culture conditions as above). Temperature was adjusted 1°C/ day, and after experimental temperature was reached, individuals were maintained in treatment conditions for 10 days prior to tissue collection (after Higuchi et al. 2015 for *Acropora* corals).

After the acclimation period, anemones were placed into cryovials and immediately placed into a -80°C freezer until processing. Frozen animals were divided into 0.2-0.5g portions and placed in 5 ml vials first filled to the 4 ml mark with glass beads (BioSpec Products, Inc., Bartlesville, OK, USA) and then homogenization buffer was added to the 4 ml mark (0.1M sodium phosphate buffer, 75 uM MgSO4, 0.15% (w/v) polyvinyl pyrrolidone, 0.2% (v/v) Triton-X-100, DI H_2O). Vials and beads were kept frozen at -20°C until addition of homogenization

buffer and tissue. Vials were placed in a bead beater (Mini Beadbeater-16; BioSpec Products, Bartlesville, OK, USA) for 1 minute and then placed in -20°C freezer for 2 minutes to keep samples cold but not frozen, with this process repeated 3 times until tissue was fully homogenized based on visual inspection of vials. Samples were centrifuged at 8500 x g for 4 minutes at 0°C (Allegra X-30R; Beckman Coulter, Brea, CA, USA). Supernatant was removed, placed into microcentrifuge tubes and serially diluted with homogenization buffer to achieve a final concentration of 1 mg tissue/ ml. Samples were then kept at -80°C until further processing: Optimization of sample concentration was performed via serial dilution of concentrated homogenate in which ETS activity of each diluted fraction was determined at 25°C, and plotted against homogenate concentration (mg tissue /ml homogenization solution) Data were then fit to a linear regression, and a working dilution concentration for subsequent ETS activity measurements was selected in the linear range of the relationship (i.e., where substrate concentrations were not limiting).

For measurement of the potential metabolic activity (PMA), samples were randomized by treatment group, individual and ETS incubation temperature. Frozen samples were thawed in an ice bath for at least 30 minutes and vortexed to ensure no ice crystals remained. 1.5 ml substrate solution (0.1M sodium phosphate buffer, 1.7 mM NADH, 0.25 M NADPH, 0.2% (v/v) Triton-X-100, DI H_2O) was added to glass test tubes (2 per sample and 1 blank). 0.5 ml INT (2.5 mM INT) was added to each tube, and 0.5 ml diluted homogenate to each sample tube with the exception of blanks. Tubes were incubated in a water bath at the proper temperature according to the randomization (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48 °C) for 30 mins. Tubes were removed from the bath, 0.5 ml stopping solution (equal parts 85% phosphoric acid and formaldehyde 37% wt:vol solution in water stabilized with 5-15% methanol) was added to

all tubes, and 0.5 ml diluted homogenate added to each blank. Samples were then poured into cuvettes and read at 490 nm on a spectrophotometer (VWR UV-1600PC) no more than 30 minutes after stopping solution was added.

PMA was calculated using the following formula (Kenner and Ahmed 1975):

$$PMA\ (ml\ O2\ g^{-1}\ WM\ h^{-1}) = ((ABS^{490nm} \times V_r \times c \times 1000)/(V_a \times S \times t \times 1.42)) / 1000,$$

where ABS^{490nm} is corrected absorbance, V_r is reaction mixture volume (3 ml), c is the correction to hours of time, V_a is the homogenate aliquot (0.5 mL); S is tissue concentration (mg/ml), t is incubation time (min), and 1.42 is the O_2 conversion factor, in units of ml O_2/ g wet mass/ h.

Data analysis

All statistical analyses were conducted using R Studio (R Core Team 2019) and SigmaPlot (Systat Software, Version 14.0). Respiration, photosynthesis, and ETS data were analyzed using ANOVA followed by Tukey post-hoc pairwise comparisons to determine significant differences by acclimation temperature.

RMR, NPR, and PMR data were analyzed in SigmaPlot against acclimation temperature (RMR), acclimation temperature and light level (NPR), and acclimation temperature and ETS assay temperature (PMR) to create thermal performance curves (TPCs). Each generated TPC was fitted with a nonlinear quadratic regression with best fit determined by R^2 and AICc values. Regression data was used to determine T_{opt}; NPR datapoints were used to determine compensation intensity, photosynthetic saturation, and photoinhibition. Aerobic scope was calculated by subtracting PMR datapoints from RMR for temperature treatments of 18, 25, and 32°C after converting RMR from mg O_2/g/hr to ml O_2/g/hr..

Results

Respiration vs Dissolved Oxygen

The amount of oxygen present in the respiratory chamber had a significant effect on the rate of oxygen consumption (RMR) in that lower levels of environmental oxygen led to decreased oxygen consumption (Fig. 3.1).

Respiration vs Temperature

Resting metabolic rate (RMR) of the anemone plus symbiotic algae in the absence of light varied significantly with temperature (Fig. 3.2A; ANOVA, $F= 244.6$, $p<0.001$). RMR did not significantly differ among the three lowest temperatures ($p=0.87$-0.995, post-hoc pairwise comparisons). However, RMR increased significantly as acclimation temperature increased to 28, and then to 32°C ($p<0.001$ for all comparisons). RMR at 28°C was ~3x higher, and ~32°C was 4 x higher than at the three lower temperatures ($p< 0.001$ for all comparisons. Unfortunately, in higher temperature trials of 28 and 32°C, dissolved oxygen concentration was not maintained at the targeted >80% saturation due to inadequate flush period that did not fully replenish the chambers with fully oxygenated water between sequential loops. At 28°C hypoxic conditions (<4 mg O_2/L) were seen in some runs, and at 32°C runs were all hypoxic. Because RMR decreased with declining DO (see Fig. 3.1)) it is likely that RMR was underestimated at 28 and 32°C, and the true increase in RMR at the two highest temperatures was even greater than that shown in Fig. 3.2A.

Photosynthesis

Net photosynthetic rate (NPR), or oxygen production (also indicated as MO_2) by symbiotic algae, also varied significantly with temperature (Fig. 3.2B; ANOVA, $F= 299.9$, $p<0.001$) as well as irradiance (Fig. 3.4). Temperature had a significant effect on photosynthesis. At the lower values for light intensity (50-150 mmol photons m^{-2} s^{-1}), photosynthetic rate did not differ in anemones at the lower temperatures (18-25°C; $p>0.05$); however, photosynthesis decreased significantly at 28 and 32°C ($p<0.001$), dropping below the net respiratory rate and resulting in net consumption of oxygen by the anemone except for the combination of 150 mmol photons m^{-2} s^{-1} and 28°C. However, as with respiration runs, dissolved oxygen concentration did not remain at >80% in 28 and 32°C treatments, suggesting we underestimated the observed NPR deficit at high temperatures.

The trend of no significant difference in rate at lower temperatures broke down at higher irradiance values, with 250 mmol photons m^{-2} s^{-1} being a transition irradiance in which all groups differed significantly from each other ($p<0.001$), with the exception of 18 and 25°C ($p=0.084$). At this light level, the highest photosynthetic rate was seen at 22°C. For the higher irradiance values (350-550 mmol photons m^{-2} s^{-1}) the general trend was for all groups to differ from one another ($p<0.05$), with some exceptions in the lower temperatures ($p<0.05$) and with the highest rate of photosynthetic MO_2 occurring at 22°C. At 650 mmol photons m^{-2} s^{-1} and above, again, all treatments differed from each other ($p<0.001$), with some exceptions in the lower temperatures ($p>0.05$); however, for these PAR values peak photosynthesis rates were seen at either 18°C or at some combination of 18 and either 22 or 25°C.

NPR increased with PAR up to a maximum oxygen production level, however, higher irradiance reduced NPR in all treatment groups. Nonlinear regression analysis generated thermal performance curves (TPCs) for each temperature treatment to allow estimation of optimum

irradiance to achieve maximum NPR at a given temperature (Fig. 3.4). The overall highest NPR was seen at 21.554°C at an irradiance of 401.563 mmol photons m^{-2} s^{-1}. Increased acclimation temperature (28 and 32°C) significantly reduced the irradiance for NPR to around half the amount of light vs that at the lower temperature treatments. Compensation intensity, where net oxygen consumption-production equals zero, was also influenced by temperature in that anemones at 28°C required ~2-3x as much light to reach the break-even point as did those at the three lowest temperature treatments. The 32°C group never reached positive oxygen production levels, but the smallest oxygen consumption level was seen at a higher irradiance level of ~300 mmol photons m^{-2} s^{-1}.

P:R

The ratio of NPR to RMR, or the P:R ratio, was significantly altered by temperature with the highest P:R ratio, >2.5, being seen for the 22°C treatment (Fig. 3.2C, ANOVA, $F=24.117$, $p<0.001$). Post-hoc pairwise comparisons showed significant differences between 18°C and 22, 28 and 32°C ($p= 0.017$, 0.001, and <0.001, respectively). Ratios for 18 and 25°C were also high (~1.5). P:R ratio was significantly reduced at higher temperatures: only >0.1 for 28°C and having a negative value (net oxygen consumption) for 32°C. However, the P:R ratios at 28 and 32°C did not significantly differ from each other ($p=0.947$).

ETS

Acclimation temperature had a significant effect on potential metabolic activity (PMA), measured via electron transport system (ETS) assays (Fig. 3.5). Thermal performance curves (TPCs) were generated for each temperature treatment via significant nonlinear regressions to

allow for estimation of maximum PMA at a given temperature. Treatment temperature affected the overall value of PMA (ANOVA, $F= 30.020$, $p<0.001$); post-hoc pairwise comparisons revealed that PMA was significantly higher at 25 and 32°C than at 18°C ($p<0.001$ for both comparisons) but PMA at 32°C was not significantly higher than at 25°C ($p=0.063$). Acclimation temperature also affected the optimum temperature for maximum PMA in that T_{opt} was lower at 31.125°C in the 18°C group when compared to 33.234°C in the 25 and 32°C groups. Additionally, aerobic scope was higher for the 25°C group, ~1.0 than the 18 and 32°C groups, <0.5.

Discussion

General Comments

These results demonstrate that acclimation temperature affects multiple aspects of the sea anemone *Bartholomea annulata*'s physiology in terms of respiration rate, photosynthetic rate, and enzyme function. This is the first study to report optimal temperature and light intensity for photosynthesis in this species. Peak net photosynthesis for this species occurs at 21.5°C, however natural field maxima for the majority of the year (March through November) are above 22°C throughout the Caribbean (Colombara and Chadwick unpublished data). Measured optimal light level of ~400 μE approaches the maxima of levels seen on Caribbean coral reefs, but may not be reached in all areas (Lesser 2000).

Respiration, Photosynthesis, and Irradiance

Basic respiratory patterns reveal that *B. annulata* most closely resembles an oxy-conformer, as is known for other species of cnidarians (Mangum and Van Winkle 1973, Shick 1990). Anemones have low oxy-regulatory ability due to reliance on simple diffusion through thin diploblast tissue layers, and thus respiration rates are heavily reliant on the amount of oxygen available for uptake in the surrounding medium (Shick 1990). In spite of inadvertent hypoxic conditions for respiration and photosynthetic trials occasionally for 28°C and entirely for 32°C treatments, observed patterns of low to negative NPR and P:R at high temperatures are likely accurate. The true NPR and P:R values are likely even lower than reported since RMR was underestimated at the high temperatures due to hypoxia. As an oxyconformer, anemones would have had even higher respiration rates if dissolved oxygen concentrations remained >80%.

In this study, respiration rate increased with temperature, as has been demonstrated for many anemone species (Verde and McCloskey 2007, Griffiths 1977) and other cnidarians such as *Cassiopea xamachana* (Verde and McCloskey 1998), and corals (Coles and Jokiel 1977; Paradis et al. 2019). Measuring respiratory and photosynthetic rates for symbiotic sea anemones *in situ* can be difficult, due to behavior of isolated algal cells versus the intact holobiont of the animal and its associated microalgae. The contribution of microalgal cell respiration compared to the anemone host is generally considered negligible, as it makes up only 0.03-0.1 parts of the symbiont:host ratio (Jurriaans and Hoogenboom 2020). Additionally, freshly isolated microalgae photosynthesize at a rate nearly 6 times higher than when found in anemone tissue (Muller-Parker 1984). Symbiont type can also have significant effects on photosynthetic and respiratory rates, with some clades exhibiting rapid division rates but high energy cost for the host, while others have lower densities but contribute more photosynthate to the host anemone (Starzak et al.

2014). As such, the identity of microalgae may determine the success of the symbiosis during environmentally stressful periods (Hill et al. 2014).

In this study, a peak was seen in net photosynthetic rate, with decreased performance above and below 22°C. Overall photosynthetic rates fall within those seen in other anemone and coral species. Harland and Davies found a gross photosynthetic rate of 1.294-1.905 ml O_2 g^{-1} hr^{-1} and respiration rate of 0.307-0.580 ml O_2 g^{-1} hr^{-1} for *Anemonia viridis*. Muller-Parker (1984) found a respiration rate of 8.18 μg O_2 mg^{-1} host protein and maximum net photosynthetic rate of 26 μg O_2 mg^{-1} host protein in *Aiptasia pulchella* (average 3.69 mg total protein per animal). Compensation intensity was low for *A. pulchella* at 32 mmol photons m^{-2} s^{-1} (Muller-Parker 1984) when compared to levels seen in this study of 55.664 mmol photons m^{-2} s^{-1} for 22°C acclimated individuals and as high as 192.968 mmol photons m^{-2} s^{-1} for the 28°C group. Muller-Parker (1984) also found that respiration rate was ~1.5 times higher after anemones were exposed to high light levels, indicating that timing of measurements and experimental design used for intact sea anemones are significant. In this study, the respiration rate was measured prior to photosynthetic rates. However, step-wise increase of light levels from 0-950 mmol photons m^{-2} s^{-1} rather than a fully randomized design may have contributed to higher dark respiration rates throughout photosynthesis trials. Higher respiratory rate following high light exposure also leads to an increase in CO_2 within anemone tissues that can allow for enhanced photosynthesis by zooxanthellae (Harland and Davies 1995). Later studies indicate that photosynthetic capacity of zooxanthellae is carbon limited, supporting this observation (Suggett et al. 2012). Similar patterns were observed in *Aiptaisa pallida* (Goulet et al. 2005); as temperature increased, compensation intensity increased from 25.5-36.5 mmol photons m^{-2} s^{-1} at 25°C to 45.7-314.9 mmol photons m^{-2} s^{-1} at 34°C. Additionally this species demonstrated loss of at least half of their

photosynthetic capacity at higher temperatures. Fitt et al. 1982 investigated photosynthetic-irradiance effects, finding that light saturation for *Anthopleura elegantissima* in intertidal areas of California reached light saturation at ~235 mmol photons m^{-2} s^{-1} with inhibition not observed until after 1550 mmol photons m^{-2} s^{-1}, which is indicative of high light tolerance. In this study, photoinhibition was seen after the peak of optimal light levels at each acclimation temperature. This optimum light level and following decrease in NPR was lower with increasing acclimation temperature, indicating potential damage to photosystems at high temperature.

The P:R ratio was highest at 22°C, indicating the largest energy surplus, while the P:R ratio of <1 at temperatures of 28 and 32°C result in reduced energy available from photosynthesis for maintenance of the holobiont (Coles and Jokiel 1977). These results are similar to a recent study on *Acropora cervicornis* corals, which saw P:R ratios of <1 for temperatures above 28°C (Paradis et al. 2019). For stony corals such as *A. cervicornis* that rely more nutritionally on their microalgae, a breakdown of photosynthetic efficiency can lead to starvation and death of the animal host. For sea anemones, which maintain higher rates of heterotrophy (Berschneider and Muller-Parker 2008), the loss of microalgae (bleaching) may not be fatal. They can recover from extended periods of time at 32°C (Colombara and Chadwick 2020), but this could contribute to additional loss of photosynthetic efficiency.

ETS Activity

In terms of respiratory enzyme function, acclimation temperature had a significant effect. The ETS method addresses many of the issues associated with traditional measurements of oxygen consumption or production such as confinement of an animal within a chamber, or the requirement to relate rates to body size measurements that can be influenced by a variety of

environmental factors; it can also be performed rapidly. As the electron transport system is tied to oxygen consumption, specific enzyme reaction rates can be used to estimate this process and represents the rate of oxygen consumption if all enzymes within the system are functioning at their maximum rate.

Thermal acclimation of enzymes plays an important role in an animal's physiological response to thermal stress. Enzymes are adapted to the specific species and environmental temperature, and deviation from normal temperatures may cause changes in enzymatic activity. Animals adapted to cold temperatures possess enzymes that have a lower activation energy, allowing metabolic reactions to proceed at faster rates in spite of the cold (Hochachka and Somero 1984). At high temperatures, enzymes begin to malfunction due to the breakdown of the tertiary and quaternary protein structures. The weak non-covalent bonds that create tertiary and quaternary structure are sensitive to temperature shifts, and the bonds tend to break, which can be detrimental to the organism (Cossins and Bowler 1987). For an animal to cope with changing temperatures, enzyme function may be altered in terms of enzyme abundance (Sidell 1983), production of enzymes with different kinetic properties (Hochachka and Somero 1984), higher affinity of the enzyme to its substrate, shifts in rate function (shift of R-T curves), or different cofactor requirements, all of which can provide acclimation benefits (Prosser 1973; Shick 1991). Thus, the optimum temperature range for function of specific enzymes affects the geographical range of an individual, and a species as a whole. Many invertebrates due to their ectothermic nature possess enzyme systems that are able to maintain function at a wide variety of environmental temperatures. Often, thermal optima estimated from ETS data do not perfectly align with thermal parameters at the organismal level (Simčič et al. 2014). The less complex the

system, the lower the thermal sensitivity which may be due to methods of oxygen supply throughout an organisms body (Portner 2002).

Thermal performance curves (TPCs; also called thermal tolerance curves, thermal reaction norm, or thermal fitness curves) describe the rate of change in a biological function (e.g. metabolic rate, enzyme activity) of an ectotherm as a result of temperature. The characteristic shape of these curves determines behavior of that particular biological function around the peak of the curve (the optimum temperature for performance, T_{opt}). At peak function, there is a risk to the organism of decreased performance even at temperatures moderately above the optimum. If an organism exists at or near thermal maxima, small changes in environmental temperature could have detrimental effects, with tropical species being especially affected (Thomas et al. 2017). This concept is demonstrated by the mathematical principle known as Jensen's inequality (Ruel and Ayers 1999), in which the functioning of an organism at suboptimal levels is actually beneficial (Martin and Huey 2008). There is the potential for an individual to cope with changing temperatures to a degree by phenotypic plasticity that may influence the height, width, or peak of a performance curve (Angilletta 2009). Determining the degree of thermotolerance is of great importance for species survival under future climate change scenarios (Sinclair et al. 2016).

The T_{opt} for ETS function in this study was not affected by the acclimation temperature in that there was no significant difference between treatment groups. However, overall level of activity was significantly higher in the 25 and 32°C groups than the 18°C group, with the former not significantly differing from each other. This indicates that anemones living close to thermal optima in the field (~25°C) are operating at nearly maximum levels of ETS activity. When regressions for PMA and RMR are compared, the maximum distance between curves occurs at 25°C, indicating the largest value of aerobic scope. When considered alongside data from

Colombara and Chadwick (unpublished observations), this window aligns with maximum growth rates at temperatures from 22-28°C, with declines seen at sublethal and lethal temperatures of 14-18°C and 32-34°C.

Conclusions

At typical irradiance levels on Caribbean coral reefs, respiration in *B. annulata* increases nearly linearly with temperature, as cellular enzyme activity increases. A rapid increase in respiratory rate acts as an energetic drain on an individual, as maximum photosynthesis by zooxanthellae occurs ~22°C and declining with higher temperatures due to photoinhibition (Lesser et al. 1990). Thus, the balance of the anemone-algae symbiosis becomes energetically compromised as both members of the mutualism are respiring at high rates. Rather than contributing positively to the energy budget of the holobiont, the host may receive signals that the algae have become essentially parasitic by consuming large amounts of intercellular oxygen, a common factor in physiological changes preceding bleaching in cnidarians (Weis 2008). The data from this study indicate that anemones at moderate to high temperatures on coral reefs experience a negative energy balance that must be mitigated in order to maintain growth, reproduction, and other energetically costly activities. As such, during warm summer months anemones are operating at physiological rates close to maximum and experience decreased growth rates and may bleach, thereby contributing to an even more negative energy balance (Colombara and Chadwick unpublished data).

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Figures

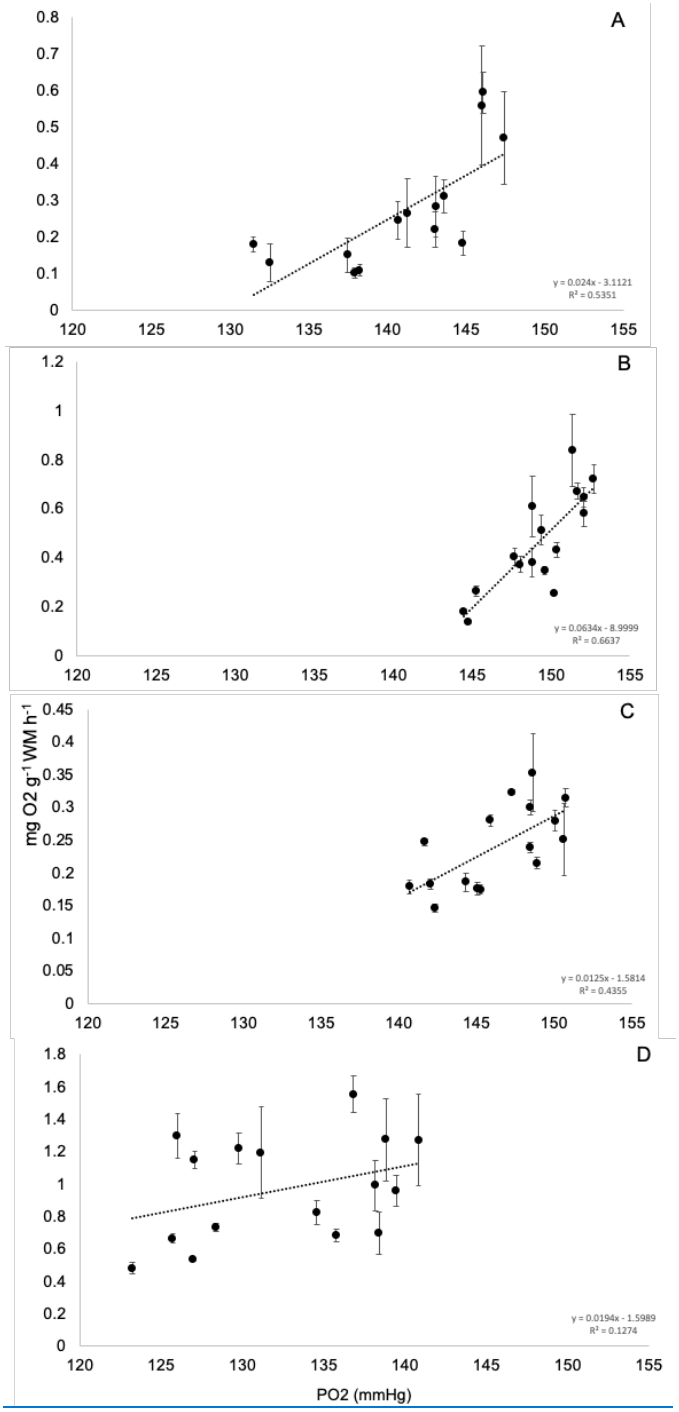

Fig. 1. Dependency of MO_2 on PO_2. Each data point represents the mean ± 1 SE for a given individual's oxygen consumption (MO_2) as a result of PO_2 and temperature treatment; A: 18°C, B: 22°C, C: 25°C, D: 28°C. Data for 32°C trial not shown here due to abnormally low PO2 values.

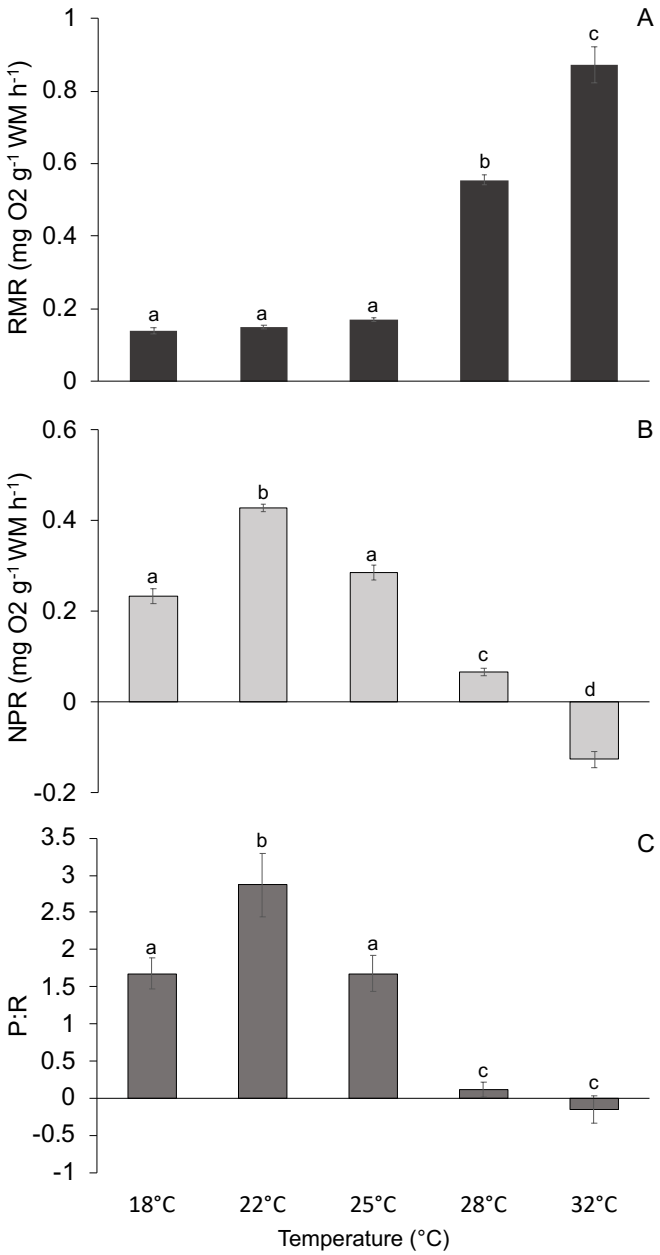

Fig 2: A: Variation in resting metabolic rate (RMR, MO_2) by individuals of *Bartholomea annulata* with acclimation temperature (N=8 individuals/treatment temperature); B: Photosynthetic rates are shown at the closest measured irradiance to optimum as determined by nonlinear regression that produces maximum net photosynthetic rate (NPR) for a given temperature (450 mmol photons m^{-2} s^{-1} for 18, 22, and 25°C, 250 for 28 and 32 °C); C: Ratio of photosynthesis to respiration, P:R. Letters above bars indicate significant difference between groups; data are presented as means ± 1 SE.

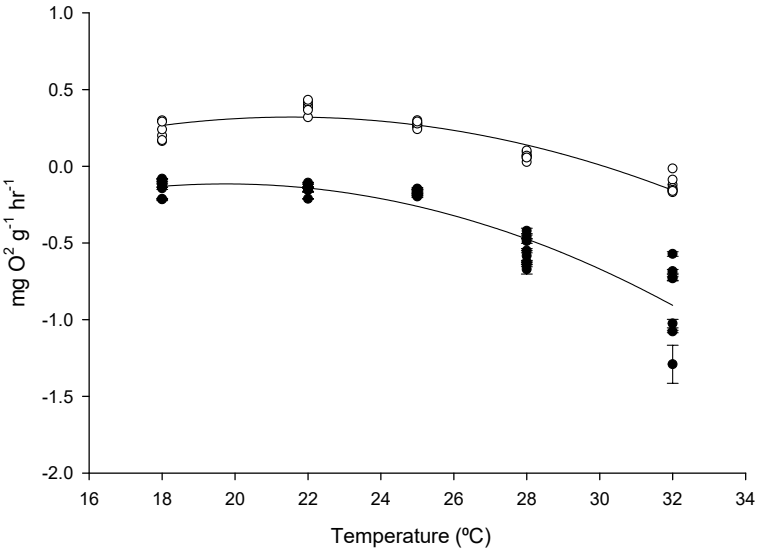

Fig 3: Thermal performance curves of resting metabolic rate (RMR) and net photosynthetic rate (NPR) in *Bartholomea annulata* for 5 temperature treatments. Each circle represents mean RMR (black) and NPR (white) for a single individual (N=8 per temperature treatment). RMR data are considered as a negative value (net oxygen consumption), while NPR is considered a positive value (net oxygen production). Lines indicate nonlinear regressions, with best fit determined by R^2 and AICc values.

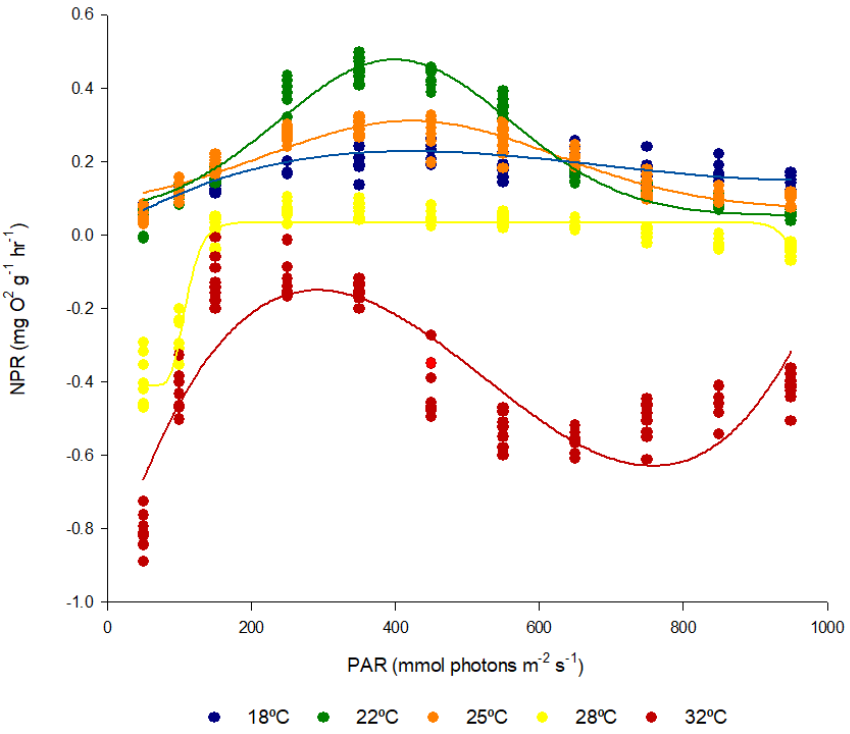

Fig 4: Thermal performance curves in *Bartholomea annulata* of variation in net photosynthetic rate (NPR, ml O_2 per gram anemone wet mass per hour) with light level (photosynthetically active radiation; PAR, mmol photons m^{-2} s^{-1}) and acclimation temperature. Each circle represents NPR for an individual, with N=8 individuals per light level per treatment for a single intermittent respirometry loop. Lines indicate nonlinear regressions, with best fit determined by R^2 and AICc values. Negative values indicate a net consumption of oxygen, as the 32°C treatment never reached compensation intensity, or positive (net oxygen production) values.

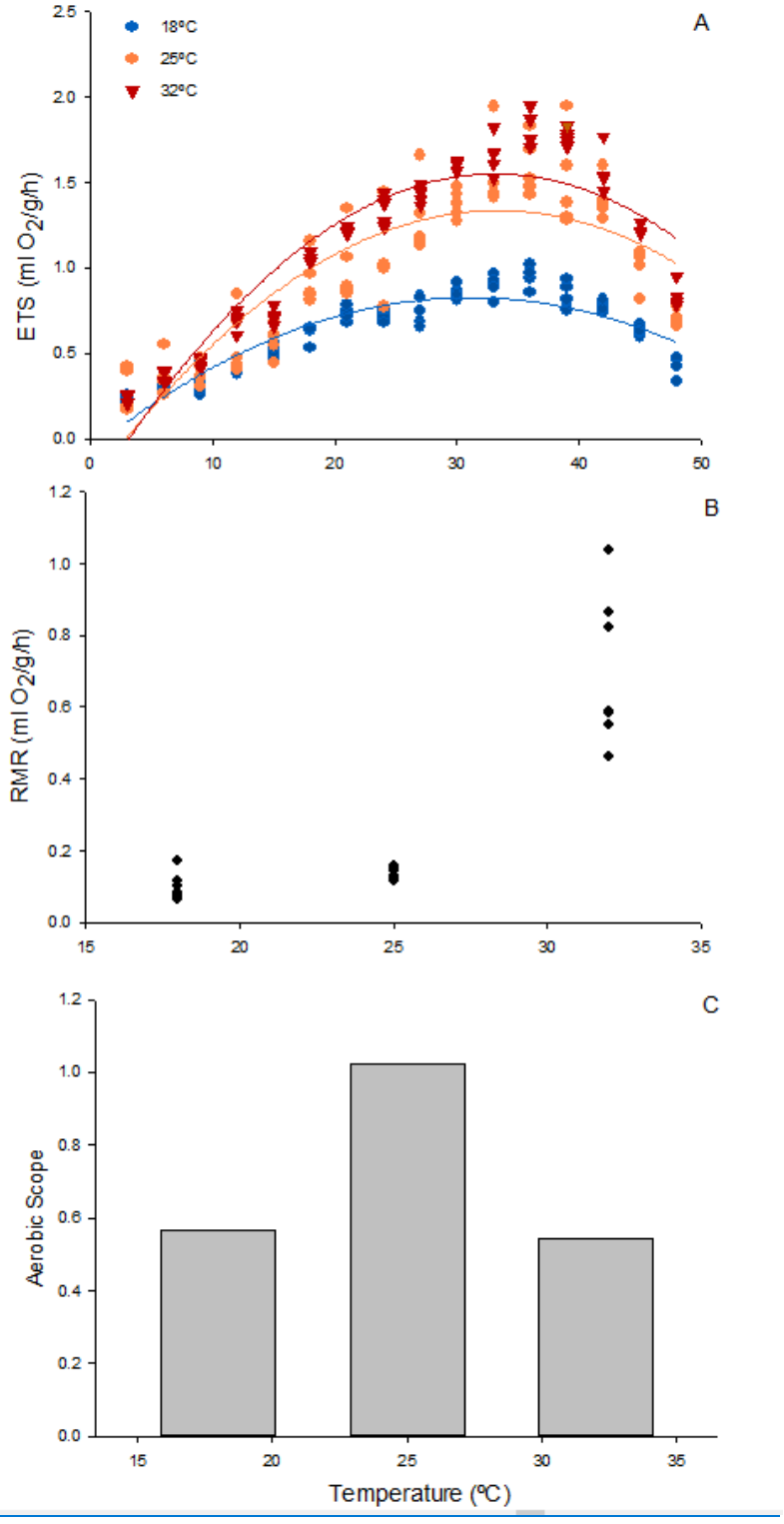

Fig 5: A: Thermal performance curves in *Bartholomea annulata* of variation in PMA by acclimation temperature. Each colored circle represents potential metabolic activity (PMA, ml O_2/ g WM/ hr) for an individual (N=8 per acclimation treatment temperature) at each ETS assay

temperature, while lines indicate nonlinear regressions, with best fit determined by R^2 and AICc values; B: Respiration rate (RMR) corrected to ETS units of ml O_2/g/hr; C: Aerobic scope estimates at 18, 25, and 32°C