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EDITED BY

Jose A. Masero,
University of Extremadura, Spain

REVIEWED BY

Patrick Buerger,
Macquarie University, Australia
Christine Schnitzler,
University of Florida, United States

*CORRESPONDENCE

Megan E. Maloney
✉ mem0294@auburn.edu

†These authors have contributed
equally to this work and share
last authorship

RECEIVED 29 March 2024

ACCEPTED 19 August 2024

PUBLISHED 09 September 2024

CITATION

Maloney ME, Buckley KM and Strader ME
(2024) Appendage pigmentation and
temperature acclimation correlate with
survival during acute heat stress in the
upside-down jellyfish, *Cassiopea xamachana*.
Front. Ecol. Evol. 12:1409379.
doi: 10.3389/fevo.2024.1409379

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Appendage pigmentation and temperature acclimation correlate with survival during acute heat stress in the upside-down jellyfish, *Cassiopea xamachana*

Megan E. Maloney^{1*}, Katherine M. Buckley^{1†}
and Marie E. Strader^{2†}

¹Department of Biological Sciences, Auburn University, Auburn, AL, United States, ²Department of Biology, Texas A&M University, College Station, TX, United States

Introduction: Upside-down jellyfish (*Cassiopea* sp.) are highly tolerant to multiple abiotic stressors, including fluctuating temperatures associated with shallow marine habitats. This resilience may underlie the ability of *Cassiopea* sp. to inhabit a wide variety of tropical habitats across the globe. Additionally, *Cassiopea* sp. are marked by a conspicuous array of appendage coloration; individual medusae vary in the hue and number of oral appendages, which are often strikingly blue. The function of this coloration is not understood. We aimed to understand how extrinsic (temperature, location) and intrinsic (host color) factors may shape thermal tolerance.

Methods: Adult *Cassiopea xamachana* were collected from two sites that vary in daily temperature range within the Florida Keys and were subjected to acute lethal heat stress. To quantify a whole-organism response to heat, we measured changes in bell pulsation, which likely plays a role in feeding, oxygen exchange, and symbiont uptake. Finally, color morphs were acclimated at either ambient (26°C) or elevated (33°C) temperatures.

Results: *C. xamachana* from two locations that vary in thermal range do not exhibit different responses to heat, suggesting that temperature fluctuations do not prime individuals for higher thermal tolerance. Additionally, *C. xamachana* with blue appendages survived significantly higher temperatures and exhibited less change in bell pulsation rates compared to non-blue individuals. We found that acclimation at 33°C, as well as appendage color in each treatment, led to higher survival under acute heat stress.

Discussion: These findings highlight the importance of temperature and coloration in *Cassiopea xamachana* resilience during heat stress.

KEYWORDS

Cassiopea, heat stress, blue coloration, acclimation, climate change, bell pulsation

1 Introduction

As global change continues to threaten marine ecosystems, rising sea surface temperature is the dominant stressor for many shallow water marine species (Filbee-Dexter et al., 2020; Lang et al., 2023; Mellin et al., 2019; Pörtner et al., 2019; Strydom et al., 2020). Ectotherms are particularly vulnerable to environmental variation, as even small temperature changes (1–2 °C; Pinsky et al., 2019) can affect physiology and behavior (Lagerspetz and Vainio, 2006; Lang et al., 2023). In response to environmental change, ectothermic organisms can adjust physiological traits (e.g., respiration rate, metabolism, and reproduction; Guderley, 1990; Seebacher et al., 2015) through phenotypic plasticity (Ghalambor et al., 2007; Hartl and Conner, 2004; Jardeleza et al., 2022). This plasticity may facilitate survival during short-term heatwave events, which can help maintain genetic diversity on which selection can act and shape the population (Botero et al., 2015; Gunderson and Stillman, 2015; Huey et al., 2012; Palumbi et al., 2019; Somero, 2010). Given the rapid pace of sea surface temperature increase, it is critical to understand the role of phenotypic plasticity in maintaining variation within and among populations of marine species, and how plasticity contributes to organismal persistence in the face of environmental stress.

Plastic phenotypes are generally favored when environmental variability is predictable (DeWitt et al., 1998; Goldstein and Ehrenreich, 2021; Moran, 1992), and the costs associated with adjusting the phenotype are minimal (Gavrilets and Scheiner, 1993; Pigliucci and Schlichting, 1998; Scheiner, 2018). As with most traits, plasticity itself can vary within and among populations, which may further affect the distribution of resulting phenotypes (Fuller et al., 2022). Natural experiments in coral reef systems indicate that individuals that live in environments characterized by high temperature variability (e.g., back-reef pools and intertidal/shallow reefs) exhibit enhanced thermal tolerance (Palumbi et al., 2014; Rivest et al., 2017; Safaie et al., 2018; Schoepf et al., 2022). Increases in thermal tolerance due to plasticity may also facilitate individual persistence in response to climate change (Schaum et al., 2022).

One of the most strikingly variable phenotypes in marine invertebrates is coloration. In general, color can be produced in one of three ways: structural coloration (i.e., microstructures that selectively interfere with light; Parker, 1998); pigments, which can be either synthesized by the organism or obtained externally; or chromoproteins (Haddock and Dunn, 2015). In some cases, color variation has been linked to physiological processes such as thermoregulation and stress response (Umbers, 2013). For example, sea cucumbers (*Apostichopus japonicus*), with either green or red coloration exhibit differential thermotolerance (Dong et al., 2010). Physiological roles for color-associated proteins have also been identified in several cnidarian species. Cnidarians exhibit particularly diverse coloration patterns, which are typically the result of either pigment-derived proteins (e.g., carotenoproteins) or fluorescent proteins (FPs; Matz et al., 2002; Shagin et al., 2004). Several *Acropora* species display polymorphism in fluorescent protein expression which have been hypothesized to contribute to acclimatization potential (Dove, 2004; Gittins et al., 2015; Jarett

et al., 2017; Kelmanson, 2003; Paley, 2014; Takahashi-Kariyazono et al., 2018). Several pigments have functions associated with regulating light available for photosynthetic algal symbionts. In reef-building corals, FPs can enhance or dissipating light, thereby modulating light available for symbionts and increasing resistance to bleaching during warming events (Salih et al., 2000). Non-fluorescent chromoproteins generated by the host can have photoprotective roles (Smith et al., 2013; Ferreira et al., 2023). However, cnidarian pigments have also been implicated in a wide variety of physiological functions, including thermal dissipation (Lyndby et al., 2016), antioxidant activity (Bou-Abdallah et al., 2006), and symbiont attraction (Aihara et al., 2019; Hollingsworth et al., 2005). Each of these could contribute to survival in stress conditions (Ferreira et al., 2023).

Studies investigating links between coloration and ecological or evolutionary processes in marine invertebrates have primarily focused on the most common colors: reds, greens, and oranges (Muñoz-Miranda and Iñiguez-Moreno, 2023). In contrast, work on blue colors has been somewhat limited, largely due to the rarity of this color in marine habitats (Lawley et al., 2021; Newsome et al., 2014). Blue coloration has been shown to affect cnidarian physiology; for example, in the scleractinian coral *Acropora aspera*, individuals with blue pigmentation maintained higher algal densities and photosynthetic efficiency compared to those that lack blue pigment in response to UV light (Dove, 2004).

One of the most striking examples of blue coloration in marine invertebrates is the upside-down jellyfish (*Cassiopea* sp.; Arai et al., 2017; Gamero-Mora et al., 2022; Medina et al., 2021; Ohdera et al., 2018). *Cassiopea xamachana* individuals exhibit a striking diversity of color variation, with many different patterns of deep blues and greens in oral appendages and throughout the mesoglea. This unique blue is produced by a chromoprotein known as Cassio Blue (Phelan et al., 2006), which has orthologs in several scyphozoan corals and jellyfish (Bulina et al., 2004; Lawley et al., 2021). Cassio Blue is highly expressed in *C. xamachana*, comprising up to 6% of the total animal protein (Phelan et al., 2006). Transition metals (Ag, Cu, Mg, Ca, Fe, and Zn) have been recovered with the Cassio Blue protein, indicating that it may serve as a ligand for metal complexation (Phelan et al., 2006). Lawley et al. (2021) suggests that the blue may serve a photoprotective function, however, physiological roles have not yet been identified for blue color or Cassio Blue in *Cassiopea* sp.

Cassiopea sp. are exceptionally robust in response to environmental variability and evidence suggests that their range is expanding globally across tropical coastal marine habitats (Bayha and Graham, 2014; Holland et al., 2004; Morandini et al., 2017; Stampar et al., 2020). *Cassiopea* sp. primarily inhabit shallow coastal ecosystems, where temperatures vary considerably among seasons and even within days (Aljbour et al., 2019; Kayal et al., 2012; Rowe et al., 2022). Studies show that *C. andromeda* are highly tolerant to stress and may even benefit from acute heat stress (Banha et al., 2020). In *C. xamachana*, this resilience appears to be independent of their symbiotic algae. *Cassiopea xamachana* that inhabit the Florida Keys are almost exclusively dominated by *Symbiodinium microadriaticum* (LaJeunesse, 2001). Although *S. microadriaticum* is generally considered to be thermally susceptible (Iglesias-Prieto

et al., 1992; Iglesias-Prieto and Trench, 1997; Warner et al., 1999), *Symbiodinium* exhibits high levels of intraspecific variation in the response to temperature (Mansour et al., 2018). Understanding the underlying physiological mechanisms that enable *C. xamachana* to thrive under temperature stress may provide unique insights into the impact of global climate change on marine invertebrates.

Despite their benthic lifestyle in shallow, low water flow habitats, *Cassiopea* sp. can generate exceptional biogenic mixing via bell pulsation, which can help facilitate ecologically important nutrient mixing within these environments (Durieux et al., 2021). Through continuous cycles of contracting and relaxing their bell, *Cassiopea* sp. can drive water and small prey around the subumbrellar cavity for feeding along the oral arms (Santhanakrishnan et al., 2012); this cyclical movement also has the potential to provide mixing of nutrients in what would normally be a low flow ecosystem (Durieux et al., 2021). Bell pulsation may also play a role in oxygen exchange and temperature regulation, such that rates of bell pulsation typically increase with rising temperatures (Arai, 1996; Béziat and Kunzmann, 2022), and decline rapidly as lethal levels are reached (Dillon, 1977). In *Cassiopea* sp., bell pulsation rates have been suggested to reflect metabolic rates (Fitt et al., 2021; Mangum et al., 1972; McClendon, 1917). Thus, this can be used as an observable, whole-organism, ecologically relevant phenotype for assessing organismal response during acute heat stress.

Here, we investigate how environmental history, appendage color, and temperature acclimation influence *C. xamachana* medusae responses to heat stress. Similarly, as in other cnidarians, we find that *C. xamachana* responds to acute heat stress by increasing bell pulsation rates until ~37 °C, at which point pulsation rates slow until lethality (38–41 °C). Daily temperature ranges that varied between collection sites had no effect on rates of bell pulsation or lethal temperature. However, we present evidence that *C. xamachana* can acclimate to increased temperatures and that survival is associated with the presence of blue appendages. This work is the first to demonstrate a link between color phenotype and resilience to thermal stress in *Cassiopea xamachana* and begins to fill the knowledge gap concerning the novel role for chromoproteins in thermal physiology.

2 Materials and methods

2.1 Collection sites and environmental data

For all experiments, *C. xamachana* medusae were collected by hand from two sites in Key Largo, FL, USA. The “Atlantic” site (25.086669, -80.453382) is near-shore and receives water flow from the Atlantic Ocean. The “Bay” site (25.079426, -80.453009) is located off docks leading into the Florida Bay (Supplementary Figure 1). Water temperature was measured at collection sites using pendant HOBO® data loggers (MX2201) were deployed from March 12 to September 9, 2022, at a depth of 1.5 m to reflect the depth at which *C. xamachana* are regularly observed. Daily mean temperatures and ranges (maximum - minimum) were

calculated using 15-minute interval data (Figures 1A, B). Statistical differences between mean daily temperature and daily temperature were determined using t-tests in R with the stats package (v. 4.3.0; R Core Team, 2023).

2.2 Experiment 1: Assessing the role of environmental variability on *C. xamachana* heat stress physiology

In May 2021, approximately 50 *C. xamachana* medusae (28–95 mm) were collected from each of the two sites (Supplementary Figure 1) and transported to Auburn University where they were acclimated for two weeks in a closed-sump aquarium system under ambient conditions (26 °C ± 1 °C; 36 ppt salinity). Animals were maintained on a 12:12 light:dark cycle and fed *Artemia* three times per week. Morphological characteristics (bell diameter, sex, and appendage morphology) were recorded, and animals were re-acclimated for one week to compensate for handling stress.

Acute heat stress trials were performed such that 10 animals were monitored simultaneously, and trials were repeated five times with different individuals (10 animals x 5 blocks = 50 total animals measured). Within each block, five individuals from each collection site were chosen at random. The evening before each trial, individual medusae were placed into 500 mL containers with mesh sides in the experimental tank (water conditions were identical to acclimation tank). Water temperature was controlled using two submersible rod heaters (Finnex Deluxe Titanium Tube Heater, 800W) connected to a digital temperature controller (SunTHIN WiFi Digital Temperature Controller 1250W, 10A). Experimental tank temperatures were monitored using pendant HOBO® data loggers (MX2201) recording temperatures at 15-minute intervals. Heat stress trials were performed at the same time each day to reduce potential variability associated with photoperiod (Nath et al., 2017). Water temperature was increased 1°C/hour; 30 minutes after each temperature increase, 1-minute videos were recorded in triplicate using Blink security cameras (Amazon) suspended above the experimental tank. Trials were complete when all medusae reached lethality, defined here as either 0.5 x baseline pulsation rate or < 10 pulses/minute. This criterion corresponded with tissue disintegration and was often accompanied by a separation of arms from bell tissue (Toullec et al., 2024). Dead animals were immediately removed from the experimental tank. Lethal temperatures and bell diameters at time of death were recorded for each individual.

2.3 Experiment 2: Testing the role of temperature acclimation on *C. xamachana* heat stress physiology

In May 2022, approximately 50 *C. xamachana* medusae of each color morph (blue and uncolored/brown) were collected from the Atlantic site (Supplementary Figure 1) and transported to Auburn University where they were randomly placed into two sump

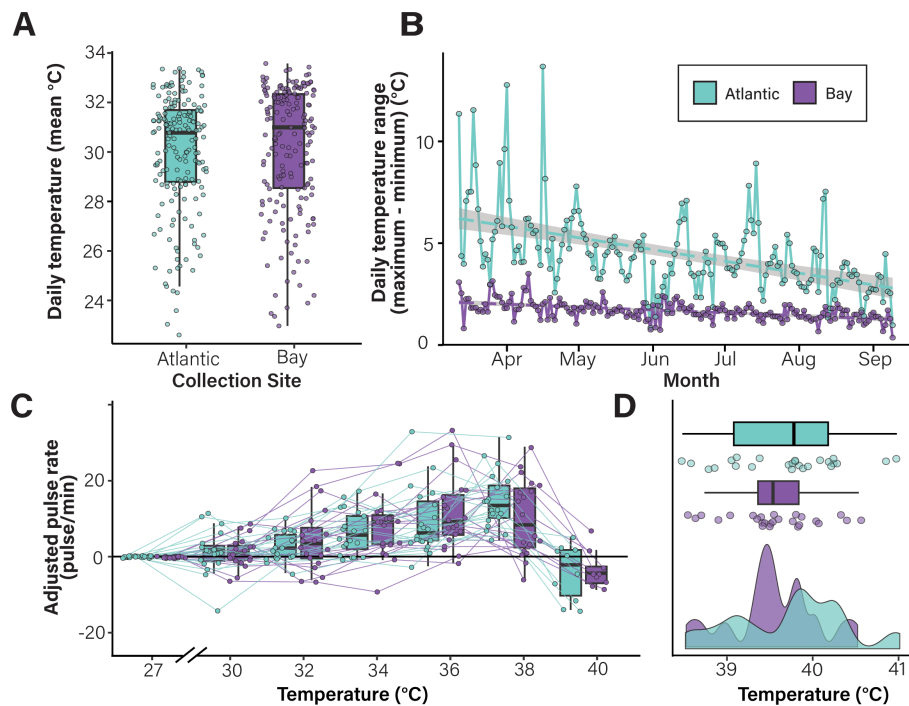


FIGURE 1

Environmental history does not affect *C. xamachana* bell pulsation rate or lethal temperature during acute heat stress. (A, B) *Cassiopea xamachana* that inhabit the Atlantic site experience greater environmental variation than those in the Bay site. Daily mean temperatures (A) and temperature ranges (B) are shown for the Atlantic (teal) and Bay (purple) collection sites. Box plots show the median value for each group as a thick line; boxes represent the interquartile range and whiskers signify the minimum and maximum values. Statistical analyses indicate that the two locations have similar daily mean temperatures ($p_{t\text{-test}} = 0.378$) but significantly variation in daily temperature (daily temperature range; $p_{t\text{-test}} < 0.0001$). (C, D) *Cassiopea xamachana* collected from the two sites do not differ in their response to an acute lethal heat stress. Each point represents data from a single individual. Data are shown as adjusted pulse rate (the difference in bell pulsation rate (pulses per minute) between the baseline temperature (27°C) and the temperature indicated). Lines connect measurements collected from individual medusa. A density plot of lethal temperatures is shown in (D) Statistical analysis indicates that collection site does not influence lethal temperature during acute heat stress ($p_{LMM} = 0.356$).

systems, each with 4 individual tanks, and maintained as described above. After a five-day acclimation period, morphological characteristics (bell diameter, sex, and appendage morphology) were recorded. Individuals were photographed at a standard height (0.5 m) using a Canon Rebel X camera with a size and color standard. Tissue samples were collected from arm tips to measure symbiont density (stored at 4°C) and isolate genomic DNA (flash-frozen in liquid nitrogen and stored at -80°C). Animals were acclimated for three days to compensate for handling stress.

The temperature in one of the two sump systems was increased 2°C per day and then maintained at 33°C (± 1 °C) for 30 days (N = 43; these animals are referred to as “elevated”). Individuals in the remaining tanks (N = 42) were maintained in ambient conditions (26 °C ± 1 °C). Temperatures were monitored using pendant HOBO® data loggers (MX2201). After the acclimation period, bell diameters were measured, and arm tissue was sampled to quantify symbiont density (stored at 4°C).

Acute heat stress trials were performed on medusae three days after post-acclimation measurements. Trials were performed as described in Experiment 1, with the additional parameters that 1) animals of each color morph were evenly distributed among the blocks; and 2) “elevated” animals were transferred to the experimental tank when the temperature matched that of their acclimation tank (33 °C).

2.4 Bell pulsation analysis

Rates of bell pulsation were quantified using the videos captured during acute heat stress experiments. Videos were viewed once per individual within the camera view. Pulsation rate was calculated as the number of pulses per minute. Bell pulsation rates vary uniquely among *Cassiopea* sp. individuals (Rowe et al., 2022). To account for this variation and assess the influence of heat stress on bell pulsation, baseline pulsation rates were calculated in each individual in the absence of any experimental manipulation. For all experiments in 2021 and the medusae that were acclimated at ambient temperatures in 2022, baseline rates were calculated after the animals were transferred to the experimental tanks and allowed to acclimate overnight to account for handling stress. In medusae acclimated at elevated temperatures (2022 trials), baseline rates were calculated in the experimental tank at 32°C. For all data shown, the individual baseline pulsation rate was subtracted from the pulsation at each temperature. All raw data, including the baseline pulsation rates and un-adjusted rates are available at <https://github.com/mem0294/Maloneyetal2024>.

To test the effects of collection site or acclimation status, appendage color, and temperature on unadjusted bell pulsation rates, a generalized linear mixed-effect model (GLMM) with a Poisson link-log function was conducted in R using the package

lme4 (v.1.1-7, Bates et al., 2015). In these designs, the location or acclimation state, appendage color, and temperature during heat stress were fixed effects, and individuals and the date of the experiment (block) were coded as random effects. To test the effects of location or acclimation, appendage color, and heat stress temperature on the change in bell pulsation rates from baseline rates, a linear mixed effect model (LMM) was conducted with the R package *lme4* (v.1.1-7, Bates et al., 2015). A LMM was used to examine differences in lethal temperature between location/acclimation and appendage colors. In this design, location/acclimation and appendage color were fixed effects; the date of the heat stress trial (block) was a random effect.

For all analyses, assumptions of normality and homogeneity of variance were graphically assessed using histograms, residual plots, and quantile-quantile plots. When there was a significant main effect ($p < 0.05$), Tukey's *post hoc* mean comparisons were conducted using the *multcomp* package (v.1.4-25, Hothorn et al., 2008).

2.5 Analyzing color, saturation and morphology of medusae appendages

Images taken before the acclimation treatment were analyzed in Adobe Photoshop CC to quantify number of pigmented appendages, color information, and blue coverage (percent). The number of pigmented appendages was quantified using FIJI (Schindelin et al., 2012) using the "Cell Counter" plugin (O'Brien et al., 2016). Appendages were counted if they met any of the following criteria: 1) contained a color dissimilar to that of the bell or frilled sections (this includes but is not limited to: blue, green, yellow, purple) or 2) morphologically matched that of a typical "large vesical" (as described in Hummelinck, 1968). The presence of "pigmented" appendages was not used to distinguish blue vs. brown individuals.

RGB (red, blue, and green) and HSB (hue, saturation, brightness) values were quantified using the Color Sampler tool. Hue refers to true color (e.g., red, green, or blue) and is measured as degrees on a color wheel, such that 0° (and 360°) is red. Saturation is the intensity or richness of color and is measured by a percentage, such that 0% indicates "no color" and 100% indicates "intense color". Brightness is also measured as a percentage such that 0–100% corresponds to black–white. Four measurements were recorded for each individual: 1) a pigmented vesicle (if present); 2) a non-pigmented oral arm appendage; 3) the bell tissue; and 4) the blue color standard that was included in each image and served as a control for variation in imaging. To normalize across images, the HSB values of the blue color standard were subtracted from each of the jellyfish measurements. A Pearson correlation was conducted in R to assess the relationship between lethal temperature and adjusted saturation percent.

The coverage of blue appendages present in individual medusae was quantified using the number of pixels (in percent) associated with blue in the R package *countcolors* (v.0.9.1, Weller and Westneat, 2019). Image backgrounds were removed in Adobe

Photoshop and replaced with white; images were manually edited to ensure that the entire medusa was included. The *countcolors* package quantifies all pixels in an image according to RGB values translated to a 0–1 scale, rather than the traditional 0–255 (Weller and Westneat, 2019). The package was run as follows: ignore white (RGB [1, 1, 1]) to exclude the background pixels and identify the "blue" pixels (RGB [0.33, 0.30, 0.25]) with an extended radius of 0.1. This color range was determined by quantifying RGB values for pigmented appendages sampled across all blue jellyfish and averaging the value. Note that, because these organisms are not bleached, the background tissue color is light brown due to the presence of the algal endosymbionts. Therefore, the color values of the pigmented appendages fall within a brownish gray, rather than pure blue. The *countcolors* package subsequently exports a new image in which pixels within the range are masked as magenta (RGB [1, 0, 1]). The accuracy of the masked images was verified manually and adjusted as necessary. Finally, *countcolors* was used on the masked images to calculate the percentage of magenta pixels in the image. A Pearson correlation was conducted in R to assess the relationship between lethal temperature and amount of blue coloration.

2.6 Quantifying symbiont density

Medusa arm tip pieces were added to pre-weighed 1.5 ml centrifuge tubes and weighed to determine wet mass. Frozen tissue was homogenized in 500–700 μL of artificial seawater (Instant Ocean) using a disposable pestle and an electric homogenizer. To separate host tissue from the *Symbiodiniaceae* cells, homogenized samples were centrifuged at $800 \times g$ for 10 minutes. Algal pellets were washed in 500 μl artificial seawater, vortexed to mix and re-homogenized as above. Samples were washed three times in artificial seawater ($400 \times g$ for 10 minutes) and resuspended in 150 μl Z-Fix (ANATECH). Symbionts were counted using an Improved Neubauer hemocytometer and normalized to the wet mass to calculate density. Eight counts were performed for each individual. A repeated measures ANOVA in R was used to examine symbiont density before and acclimation. In this design, acclimation treatment and appendage color were categorized as fixed effects; individual medusa ID was used as the repeated measure, and therefore the random effect.

2.7 Verifying *Cassiopea* species

DNA from arm tissue was extracted using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek) according to manufacturer's instructions. DNA concentrations were quantified using the dsDNA Broad-Range Qubit assay (Invitrogen). The mitochondrial gene cytochrome *c* oxidase subunit I [COI] was amplified using custom-designed primers (Supplementary Table 1; Ye et al., 2012). Thermal cycling conditions were 3 min at 95°C for initial denaturation, followed by 35 amplification cycles (denaturation at 95°C for 35 s, annealing at 49°C

for 40 s and extension at 72°C for 50 s) and a final extension for 7 min at 72°C. Amplified products were cleaned (Zymo Research Genomic DNA Clean and Concentrator-10 kit) and quantified using the dsDNA Broad-Range Qubit assay (Invitrogen). Purified products were sequenced using Sanger chemistry with custom primer MM1 (Supplementary Table 1). COI sequences were aligned to publicly available sequences from *C. xamachana* (Genbank accession NC_016466.1; Ohdera et al., 2019) and *C. andromeda* (OP503353.1 and OP503325.1; Kayal et al., 2012) using Geneious v. 2022.2.2.

2.8 Identifying Symbiodiniaceae type using ITS2 amplicon sequencing

Medusae collected from the Bay (n = 6) and Atlantic sites (n = 5) were used to characterize symbiont communities. Tissues samples were collected from arms, stored in ethanol and used for DNA extractions as described. ITS-2 regions were amplified using GoTaq MasterMix (Promega), and Symbiodiniaceae-specific primers (final concentration 400 nM) with adaptors for Illumina sequencing (Supplementary Table 1). Reactions were amplified as follows: 95°C for 3 min, 25 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. Samples were purified with the Genomic DNA Clean and Concentrator-10 kit (Zymo Research) and sequenced on the Illumina MiSeq platform (150 bp paired-end reads; Georgia Genomics).

The R package dada2 (Callahan et al., 2016) was used to identify and quantify the Symbiodiniaceae taxa present in each sample. After removing chimeric sequences, valid amplicon sequence variants (ASVs) were identified and enumerated. To identify the proportion and identity of the symbiont community in each sample, we created a BLAST database containing annotated ITS2 sequences from the SymPortal framework (<https://symportal.org>).

3 Results

3.1 Animals used in this study are *C. xamachana*

Given the morphological variation and potential for cryptic species among *Cassiopea* sp., (Muffett and Miglietta, 2023), the species identity of collected animals was confirmed. DNA isolated from medusae used for the 2022 acute heat stress experiment was used to amplify the COI gene for sequencing. All 30 sequences exhibited >99.6% sequence identity with the *Cassiopea xamachana* COI sequence (Kayal et al., 2012; Supplementary Figure 2A). Further, 11 representative animals from the collection sites were selected for ITS-2 symbiont typing. These medusae were overwhelmingly dominated by *Symbiodinium A1* (Supplementary Figure 2B), which is consistent with studies showing strong host-symbiont fidelity between *C. xamachana* and *S. microadriaticum* (LaJeunesse, 2017). Thus, despite the observed morphological variation among individuals, medusae used in these studies are *C. xamachana*.

3.2 Daily temperature variation does not affect rates of bell pulsation or lethal temperatures during acute heat stress

To determine if environmental history affects how *C. xamachana* responds to heat stress, medusae were collected from two sites that vary significantly in daily temperature range (“Atlantic” and “Bay” sites; Supplementary Figure 1). Temperature data collected from the two sites revealed that, although these locations had similar average daily temperatures (30.1°C in the Bay and 29.9°C in the Atlantic; Figure 1A), the Atlantic site water temperatures were significantly more variable than those in the Bay site ($p_{t-test} < 0.001$, Figure 1B). On average, water temperatures at the Atlantic site varied 4.55°C per day compared to 1.73°C at the Bay site. This variability may reflect changes in water depth due to tidal influx.

Animals from each site were exposed to an acute lethal heat stress (starting from ambient temperature and increasing 1°C/hour until lethality). Bell pulsation rates were used to monitor organismal response. Notably, no significant differences were observed in the change in bell pulsation rates among animals collected from the two sites (Figure 1C; Supplementary Figure 3; Supplementary Table 2). The rates of bell pulsation increased ~3.1 pulses per minute for each 1°C increase in water temperature ($p_{GLMM} < 0.001$, Supplementary Table 3) until 37°C, at which point pulsation rates dropped dramatically just prior to mortality. *Post-hoc* analyses identified statistical differences in the change in pulsation rates at several temperatures relative to baseline (Supplementary Table 4). Additionally, no significant difference was observed in average lethal temperatures of *C. xamachana* collected from the Atlantic (39.81°C), and Bay sites (39.52°C; $p_{LMM} = 0.356$, Figure 1D, Supplementary Table 5). Notably, despite surviving to 40°C, we observed no evidence of symbiont loss (bleaching) in medusa from either collection site. Together, these data indicate that, despite the difference in daily temperature range and variability between collection sites, *C. xamachana* from both locations displayed similar physiological phenotypes in response to acute heat stress.

3.3 Temperature acclimation influences bell pulsation and lethal temperature

To determine if *C. xamachana* can acclimate to elevated temperatures, medusae (N=100) were collected from the Atlantic site and maintained at either ambient (26°C) or elevated (32°C) temperature for 30 days (Supplementary Figure 4A). The different acclimation temperatures did not cause significant differences in bell diameter or symbiont density (Supplementary Figure 4B, C). In fact, medusae in both treatment conditions exhibited reduced bell diameters and increased symbiont density (Supplementary Figure 4B, C), which may reflect the transition to the artificial aquarium system. Following acclimation, 40 individuals ($N_{\text{ambient}} = 20$, $N_{\text{elevated}} = 20$) were randomly selected for heat stress trials.

Following the 30-day acclimation period and after the first group of animals was subjected to experimental heat stress, an

unexpected mechanical failure in the ambient tank (26°C) caused a temperature decrease of 5°C for 24 hours. The remaining individuals were thus treated as “cold-shocked”. To account for the possibility that this affected subsequent responses, shock status was added as a fixed effect for all models. Notably, no significant interaction of shock status was observed with any other treatment factors. We have therefore interpreted fixed effects (temperature, acclimation treatment, and appendage color) equally.

Animals were exposed to an acute heat stress starting from the acclimation temperature and increasing 1°C/hour until lethality. As in the initial study, rates of bell pulsation steadily increased with temperature until animals approached lethality (Figure 2; Supplementary Figure 5). Acclimation temperature had a significant effect on the change of bell pulsation rates from the start of heat stress ($p_{LMM} = 0.008$, Figure 2A, Supplementary Table 6). Notably, medusae acclimated in elevated conditions survived to higher temperatures compared to those acclimated at ambient temperatures. The average lethal temperature for *C. xamachana* acclimated at ambient temperature was 39.97°C compared with animals acclimated at elevated temperatures (41.2°C, $p_{LMM} < 0.0001$; Figure 2B; Supplementary Table 8). This data show that *C. xamachana* can acclimate to a consistent period of elevated temperatures and that this acclimation is beneficial in the context of acute heat stress.

3.4 Appendage color is associated with *C. xamachana* survival during acute heat stress

Given the lack of significant differences in medusae collected from two sites (Figure 1), we were interested in identifying other factors that might influence *C. xamachana* physiology during heat

stress. We therefore investigated if the variation in color phenotypes is associated with survival during heat stress. Using the data from both experiments, *C. xamachana* individuals were categorized as either “blue” or “brown” based on the predominant color of their oral appendages and all data were re-analyzed in the context of appendage color (Figure 3). Importantly, no relationship was observed between appendage color and either bell diameter or symbiont density (Supplementary Figure 6). The change in rate of bell pulsation during the acute heat stress experiments was not significantly different between medusae with blue appendages or brown appendages in either experimental year (2021 experiment, $p_{LMM} = 0.699$, Figure 3A; 2022 experiment, $p_{LMM} = 0.681$, Figure 3B). However, in both experiments, medusae with blue appendages survived significantly higher temperatures than those without (Figure 3C; 2021 experiment, $p_{LMM} = 0.003$, Supplementary Table 5; 2022 experiment, $p_{LMM} < 0.007$, Supplementary Table 8). This effect was also observed in medusae collected from both sites in 2021, such that individuals with blue appendages from the Atlantic site survived to significantly higher temperatures than those from the Bay site ($p_{LMM} = 0.0013$; Figure 3D; Supplementary Table 9). In both blue and brown individuals, elevated acclimation treatment was significantly associated with higher survival ($p_{LMER} < 0.0001$; Figure 3D).

To further investigate if there is a relationship between the amount of blue coloration coverage/saturation of pigmented appendages and lethal temperature during an acute heat stress, appendage morphology was quantified as the number of pixels within the average RGB of blue appendages. A significant, positive correlation was observed between the percentage of blue coverage within a medusa and lethal temperature (Figure 4A, $R = 0.39$; $p = 0.032$). Similarly, we found a significant, positive correlation between the adjusted saturation of the appendage and lethal temperature (Figure 4B, $R = 0.41$, $p = 0.024$). We however

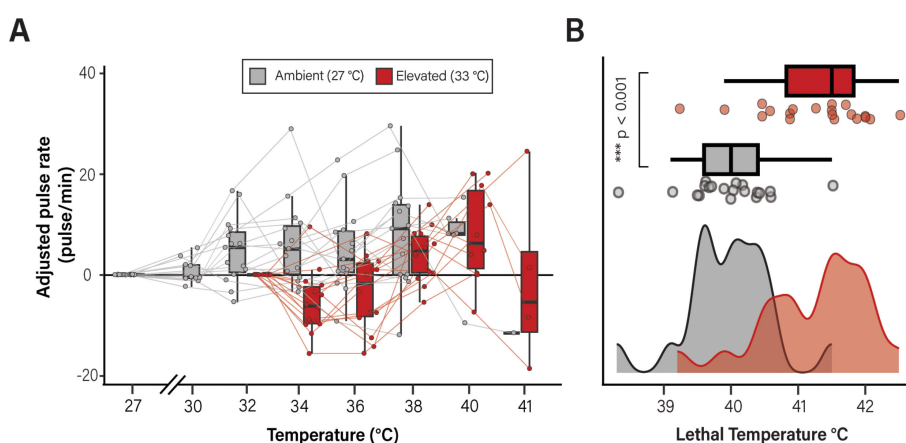


FIGURE 2

Temperature acclimation significantly affects bell pulsation rate and lethality. Individual medusae were acclimated at either 27°C (ambient; shown in red) or 32°C (elevated; shown in black) and subsequently exposed to an acute lethal temperatures stress and monitored for rates of bell pulsation (A) and temperature at which death occurred (B). Data are shown as adjusted pulse rates such that, for each animal, the baseline rate (measured at either 27°C for ambient or 32°C for elevated individuals) was subtracted from the pulsation rate at the temperature indicated. Pulsation rates are shown as pulses per minute. Lines connect measurements collected from individual medusa. Data in B are shown as a histogram of lethal temperatures during the acute heat stress trial. A significant difference ($p_{LMM} < 0.001$) was observed in the lethal temperature of *C. xamachana* acclimated at ambient vs. elevated conditions. Box plots show the median value for each group as a thick line; boxes represent the interquartile range and whiskers signify the minimum and maximum values.

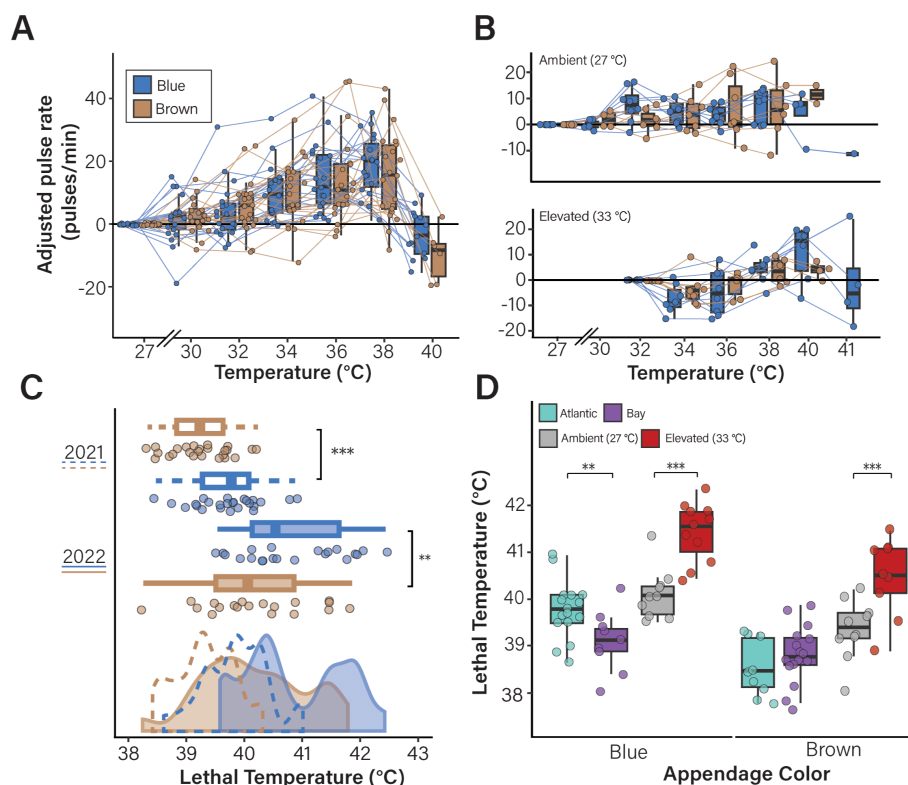


FIGURE 3

The presence of blue appendages is associated with *C. xamachana* survival during acute heat stress. Data from the experiments described in Figures 1, 2 were re-analyzed in the context of oral appendage color. Animals were classified as either "blue" or "brown" based on the predominant appendage color. No significant difference was observed in the adjusted pulsation rates of blue vs. brown animals in either experiment (2021 collection site data is shown in (A) $p_{LMM} = 0.44$; 2022 acclimation data is shown in (B) $p_{LMM} = 0.94$). Lines connect individual medusa between measurement points. Black dots indicate the final pulsation rate measured before lethality. (C, D) Appendage color affects lethal temperature during acute heat stress. Lethal temperatures in the context of appendage color are shown for the medusae collected in 2021 (C; $p_{LMM} = 0.0013$) and 2022 ($p_{LMM} = 0.004$) with significant differences denoted with asterisks (**, $p < 0.05$; ***, $p < 0.001$). (D) The effect of appendage color on lethal temperature in the context of collection location ($p_{LMM} = 0.0007$, Atlantic = teal, Bay = purple) and acclimation treatment ($p_{LMM} = 0.004$, gray = ambient, red = elevated) are shown. Box plots show the median value for each group as a thick line; boxes represent the interquartile range and whiskers signify the minimum and maximum values.

found no significant relationship between the number of pigmented appendages and lethal temperature (Figure 4C, $R = 0.26$, $p = 0.15$). Analysis of the acclimation treatment data revealed positive correlations between percent blue coloration and lethal temperature in medusae acclimated in either temperature treatment, although this effect was stronger in ambient conditions ($R = 0.55$, $p = 0.034$), relative to elevated ($R = 0.36$, $p = 0.19$, Supplementary Figure 7A). Positive correlations were also present for adjusted saturation (Supplementary Figure 7B). Representative medusa and their corresponding results for blue coverage, adjusted saturation, number of appendages, RGB and HSB are shown in Figure 4D.

4 Discussion

Here, we present results that highlight how temperature regimes and coloration shape the ability of upside-down jellyfish (*Cassiopea xamachana*) to respond to thermal stress, revealing mechanisms underlying the remarkable resilience of this species. We find that animals with different environmental history, specifically

differences in daily temperature range, do not exhibit variation in their thermal stress responses. However, short-term acclimation and the presence of blue appendages was associated with enhanced survival in *C. xamachana*. Ultimately, these findings highlight a novel link between blue coloration and resilience in this marine invertebrate.

4.1 Temperature influences the rate of bell pulsation in *Cassiopea xamachana*

Among symbiotic marine cnidarians, *Cassiopea* appears to be uniquely capable of thriving in a range of environmental conditions (Purcell, 2012; Toullec et al., 2024). Specifically, *C. andromeda* exhibits exceptional tolerance to repeated, intense (26–34°C) heat stress episodes (Banha et al., 2020; Béziat and Kunzmann, 2022). Here, using rates of bell pulsation as a measure of organismal physiology, we find that *C. xamachana* maintain homeostatic bell pulsation over a broad, but ecologically-relevant, temperature range (26–34°C). This contrasts with several species of tropical cnidarians in which bell pulsation rates change dramatically beyond a narrow

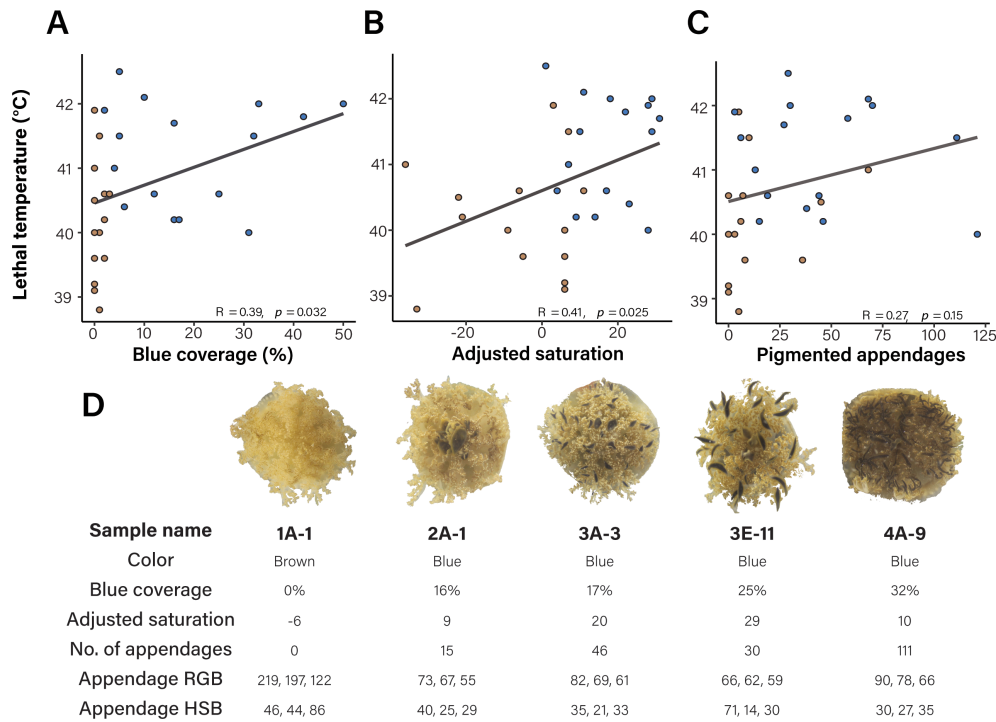


FIGURE 4

Appendage coverage and saturation correlate with survival during acute heat stress. Blue appendage coverage (A, $R = 0.39$; $p = 0.032$), and adjusted saturation (B, $R = 0.41$, $p = 0.024$) had a positive and significant association with lethal temperature in medusa subjected to an acute heat stress, while number of appendages (C) was not significant ($R = 0.26$, $p = 0.15$). Points represent individual medusae and are colored according to their manual color classification (either brown or blue), lines represent the Pearson correlation between lethal temperature and appendage metric. (D) Representative medusae with distinct morphologies are shown. For each medusa, the color morph, blue coverage, adjusted saturation values, number of pigmented appendages, RGB and HSB scores are indicated.

temperature range (Béziat and Kunzmann, 2022; Gatz et al., 1973). In *Cassiopea* sp., bell pulsations create water currents that facilitate food capture (Gohar and Eisawy, 1960; Hamlet et al., 2011; Klein et al., 2016) and oxygenate the surrounding water (Rowe et al., 2022). In the context of temperature stress, increased rates of bell pulsation may serve as a mechanism to enhance metabolic processes during stress (Fitt et al., 2021; Mangum et al., 1972; McClendon, 1917). This phenomenon has been observed in other marine invertebrates responding to acute temperature stresses (e.g., crayfish, Ern et al., 2015; gastropods, Valles-Regino et al., 2022). In response to temperature fluctuations, *Cassiopea* sp. exhibit increased superoxide dismutase (SOD) activity (McCord and Fridovich, 1969), signs of oxidative stress (Regoli and Giuliani, 2014), and altered metabolic rates (Aljbour et al., 2019; Fridovich, 1973; Lesser, 2006; McCord and Fridovich, 1969; Regoli and Giuliani, 2014). Stress conditions, such as increased temperature beyond physiological limits, may increase the demand for metabolic energy and, in turn, increase reactive oxygen species (ROS) (Boveris and Chance, 1973; Speakman and Selman, 2011). This ability to overcome oxidative stress may underlie *C. xamachana* resilience to thermal stress. Future work should aim to clarify the physiological and metabolic benefits or costs of increasing bell pulsation and how this contributes to the remarkable thermotolerance of *C. xamachana*.

4.2 Environmental history does not shape how *C. xamachana* respond to acute heat stress

It has been proposed that high environmental variability can result in increased phenotypic plasticity (Lande, 2014). This theory is supported by several empirical studies in marine invertebrates, demonstrating that organisms living in fluctuating environments (e.g., drastic fluctuations in temperature or salinity) exhibit increased resistance to temperature stress associated with climate change (Burton et al., 2022; Godefroid et al., 2023; Kapsenberg and Cyronak, 2019; Rivest et al., 2017; Safaie et al., 2018). In contrast, here we demonstrate that *C. xamachana* collected from a site with high daily fluctuations in temperature exhibit the same physiological (bell pulsation rate) and survival (lethal temperature) response during heat stress than individuals collected from a site with stable daily temperatures. Similar experiments in other cnidarian species have produced contradicting results. For example, temperature fluctuations on daily or tidal timescales were suggested to be sufficient to promote thermal tolerance of some corals via acclimation, while high temperatures were short enough to avoid lethality (Castillo and Helmuth, 2005; Oliver and Palumbi, 2011; Safaie et al., 2018). However, the effect of temperature variability on heat tolerance is likely species-specific (Putnam and Edmunds, 2011; Schoepf et al., 2022; Voolstra et al., 2020).

Here we show that *C. xamanchana* are robust to thermal stress, and that this was not likely explained by inhabiting environments with variable daily temperatures. Several potential factors may contribute to this result. First, the temperature fluctuations between the two sites may not be sufficiently different to elicit distinct physiological effects. Corals from habitats such as the back-reef pools in American Samoa experience daily temperature fluctuations up to 5.6°C, even reaching daily extremes of >35°C (Barshis et al., 2010); whereas the nearby, less-variable forereef experiences seasonal maximum daily temperature fluctuations of 1.8°C (Barshis et al., 2018; Craig et al., 2001). These corals have phenotypic differences consistent with local adaptation of thermal performance (Palumbi et al., 2014). Similarly, either the temperature or the duration of daily maximum temperature in the Atlantic site may be insufficient to induce increased thermal tolerance (either via acclimation or local adaptation). While our COI data revealed that all animals used in this study are *C. xamanchana* (consistent with Muffett and Miglietta, 2023), these data are unable to resolve population-level differences or assess local adaptation. Thus, it is possible that *C. xamanchana* sampled from both sites represent the same population and are thus robust to stable and fluctuating temperatures regardless of the site they are found. These results are consistent with the overall observed robustness of *C. xamanchana* relative to other marine invertebrates (Aljbouir et al., 2019; Banha et al., 2020; Béziat and Kunzmann, 2022; Fitt et al., 2021; McGill and Pomory, 2008; Medina et al., 2021; Ohdera et al., 2018; Rowe et al., 2022). In fact, *Cassiopea* sp. thrive in human-disturbed environments (Stoner et al., 2011, 2016) and are expanding their habitat range. Our results may therefore reflect the underlying thermal tolerance of *C. xamanchana* rather than environmental conditions of specific collection sites.

4.3 Acclimation allows *C. xamanchana* to survive elevated temperatures

As oceans warm for more prolonged periods due to global change, marine organisms must adjust their physiology to tolerate greater extremes (Coumou and Rahmstorf, 2012; Lopez et al., 2018; Morley et al., 2019). Short-term temperature stress can lead to subsequent increases in thermal tolerance in cnidarians (Baker et al., 2008; Kenkel and Matz, 2016; Kirk et al., 2018). The work presented here indicates that acclimation to a warmer environment may promote survival under acute heat stress and thus may prepare *C. xamanchana* medusae for future warming conditions. Given the rapid heating of shallow water environments, *C. xamanchana* may have an advantage if previous warming allows them to acclimate; it is possible that the previously encountered temperature “extreme” in our acclimation experiment could have buffered the effect of the acute heat stress, thereby allowing them to survive to higher lethal temperatures (*i.e.*, stress memory; Crisp et al., 2016; Li et al., 2020).

These results, while novel for *C. xamanchana*, are not uncommon among marine ectotherms. In natural experiments, corals of the Great Barrier Reef pre-exposed to thermal stress exhibited protection from subsequent heat waves (Ainsworth et al., 2016; DeMerlis et al., 2022). It is hypothesized, that an organismal thermal threshold might

be a plastic trait, in which some individuals are able to extend the upper thermal limit over time (Buckley and Huey, 2016; Leung et al., 2021). These observations are consistent with the laboratory-based experiments presented here and by others investigating cnidarian thermal tolerance (Ainsworth et al., 2016; Bay and Palumbi, 2015; Bellantuono et al., 2012a, 2012b; Middlebrook et al., 2008). *Cassiopea* appear to be resilient to temperature stress, which may be the result of a hardening capacity, or the ability to increase thermotolerance due to experiencing a prior non-lethal temperature stress (Sgrò et al., 2010). The results presented here emphasize *Cassiopea*'s ability to acclimate to warming temperatures but may also predict their invasion potential and survival in more diverse environments. Some studies have already investigated *Cassiopea*'s invasion into more temperate environments (Rowe et al., 2022), however it is possible, due to their ability to acclimate, they may expand their range into warmer habitats.

4.4 Appendage color is associated with survival in warming environments

How temperature-dependent processes differ among color morphs could impact species survival and influence how polymorphisms evolve and/or persist (Thompson et al., 2023). In the present study, we observed differences in *C. xamanchana* response to heat stress in medusae containing blue appendages compared to those lacking blue appendages. This finding was consistent across two years of experimentation using individuals collected from different sites, which demonstrates the strength of our hypothesis that pigmented appendages may benefit *C. xamanchana* during extreme heating events. This is the first record of appendage color contributing to fitness traits in *C. xamanchana*. While blue appendage color is common among some *Cassiopea* species, other color morphs are also common, including red, blue, purple, green, brown, and white (Lampert et al., 2012). *Cassiopea* sp. also vary dramatically in the level of pigmentation, appendage shape and appendage number (Lampert et al., 2012; Rowe et al., 2022). Color has been associated with camouflage, sexual selection, and social interactions in many organisms (Cuthill et al., 2017), although these roles are unlikely in *Cassiopea*. Given their habitat (shallow, clear seawater), and ecology (epibenthic, with brown coloration mimicking the substrate), bright blue coloration makes individuals more obvious. Additionally, *Cassiopea* males freely release sperm into the seawater and females brood larvae and therefore do not undergo coloration-based mate selection. Based on the results presented here, we hypothesize that the Cassio Blue protein provides *Cassiopea* with an advantage in thermal stress conditions at the level of cellular physiology.

Several hypotheses on the function of Cassio Blue have been proposed. One possibility is that Cassio Blue facilitates optimal photosynthesis via mitigation of solar radiation (Phelan et al., 2006), although Cassio Blue pigmentation does not strongly colocalize in tissue with photosymbionts. Other chromoproteins have been proposed to absorb excess light and thus optimize the environment for algal symbionts (Ferreira et al., 2023; Salih et al., 2000; Smith et al., 2013). It has also been hypothesized that the

pigmented areas in *Cassiopea* provide a refuge for the symbiotic algae and that certain colors correlate with specific algal strains. However, data presented here and by others (Lampert et al., 2012) indicate that this is not the case. Different color morphs harbor identical symbiont communities (Supplementary Figure 2), and symbionts were present in both pigmented and non-pigmented tissues. Furthermore, an ortholog of Cassio Blue is present in the blue jellyfish, *Rhizostoma pulmo*, which does not harbor symbionts, making it likely that the primary role for this protein is related to host, rather than symbiont, physiology (Bulina et al., 2004; Lawley et al., 2021).

The Cassio Blue protein is composed of Frizzled and Kringle domains, neither of which have been previously associated with pigmentation or thermal stress responses (Lawley et al., 2021). However, based on physiological roles for fluorescent proteins in other cnidarians and chemical properties of Cassio Blue, we propose two hypotheses for how this protein may promote survival in heat stress. In *Cassiopea* sp., heat stress induces signs of oxidative stress (Aljbour et al., 2019) and increased production of reactive oxygen species (ROS). To attenuate potential damage from ROS, some cnidarians express proteins with antioxidant properties. For example, fluorescent proteins expressed in several species of scleractinian corals serve as scavengers for ROS (Palmer et al., 2008). As an alternate hypothesis, Cassio Blue is associated with transition metals, including Cu, Fe, and Zn (Phelan et al., 2006). It is therefore possible that this protein serves as an important reservoir for these trace metals, which are required for metabolic processes such as the protein synthesis and enzyme activity needed for physiological acclimation (Reich et al., 2021). Metals have also been implicated in several aspects of coral physiology, specifically relating to the ability of algal symbionts to photosynthesize (Ferrier-Pagès et al., 2018; Reich et al., 2021, 2023). *Symbiodiniaceae* have higher metal requirements to initiate electron transfer between photosystems (Camp et al., 2022; Raven et al., 1999; Reich et al., 2020; Rodriguez et al., 2016). These hypotheses are not mutually exclusive; Cassio Blue may be involved in both functions. Future work will be necessary to examine the mechanistic role of Cassio Blue in *C. xamachana* physiology.

Trade-offs between fitness benefits and energetic costs of producing pigmentation may impact organismal physiology (Calsbeek et al., 2010), however these factors may not be relevant during acute heat stress in *Cassiopea*. In some organisms, selection pressures for coloration may result in variation in color traits (Zajitschek et al., 2012). However, in *C. xamachana*, blue appendages do not appear to influence whole-animal performance traits, such as bell pulsation, but may instead contribute to overall survival through alternative mechanisms (Husak and Fox, 2008; Irschick et al., 2008; Zajitschek et al., 2012). Blue color in many organisms can be achieved by combining a chromophore with an apoprotein (Bulina et al., 2004), which is not a common phenomenon due to the complex structure of blue pigments. Although the chromophore for Cassio Blue has not yet been identified, it is likely that the benefits of blue pigmentation outweigh the potential costs of producing the protein.

Color and fluorescent proteins are extremely common in Cnidaria and have been associated with immunity functions,

growth enhancement, and photoprotection (D'Angelo et al., 2012; Palmer et al., 2008, 2009; Salih et al., 1997; Smith et al., 2013). While functional studies on blue pigmentation in *Cassiopea* sp. have been somewhat limited, considerable insights can be gained from work in corals. Populations of the stony coral *Montastraea cavernosa*, which have green and red color morphs, possess the same gene, but exhibit differences in mRNA abundance between morphs (Ferreira et al., 2023; Kelmanson, 2003). According to a study by Gittins et al. (2015), copy number and expression level of the RFP gene was found to be an important factor in the intensity of red fluorescence in *Acropora millepora* (Ferreira et al., 2023). Like what has been studied in corals (Bollati et al., 2022; Dove, 2004; Lyndby et al., 2016; Salih et al., 2000), *Cassiopea* sp. appendage colors other than blue could generate different light microenvironments, which may be important for photoprotection in shallow waters (Lyndby et al., 2023).

5 Conclusions

In the present study, we assessed the impact of environmental history, temperature acclimation and appendage color on the rate of bell pulsation and survival during an acute heat stress in *Cassiopea* jellyfish. Overall, we demonstrate that *C. xamachana* medusae increase bell pulsation rates during an acute heat stress until temperatures reach ~37°C, at which point the rates sharply decline as the animals approach lethality. Surprisingly, we found that environmental history did not affect bell pulsation or survival, but pre-conditioning *C. xamachana* to warmer temperatures enables acclimation and survival to more extreme temperatures. Finally, our data show for the first time that the presence of blue appendages corresponds to survival of *C. xamachana* under warming climates. Together, these data hint at novel physiological mechanisms controlling the organismal response to thermal stress. Understanding the mechanisms that allow species to survive environmental stressors has become increasingly important as climate change continues to threaten marine taxa. This work contributes to our broader understanding of how phenotypic plasticity and color variability can shape organismal survival.

Data availability statement

The datasets generated and analyzed for this study can be found in the GitHub repository: <https://github.com/mem0294/Maloneyetal2024>.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

MM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Validation. KB: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. MS: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Funding was secured via KMB and MES through Auburn University Department of Biological Sciences start up.

Acknowledgments

We thank Emily Wilkins, Sammy Kutsch, Sarah Leinbach Cole Moquin, and Andrew Meyer for field/lab support, and Dr. Bill Fitt for housing support in the Florida Keys. Research and

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collection was completed under permits issued by Florida Fish and Wildlife.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2024.1409379/full#supplementary-material>

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