Disentangling the effects of thermal stress on symbiont-bearing coral reef foraminifera – from populations to proteins –

Dissertation by
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Bremen, den 11. Dezember 2017

Summary

Photosymbiosis is of central importance for the wellbeing and proliferation of many tropical marine organisms, but the balance within this relationship can react delicately to changes in environmental conditions. Many of such reef-building calcifiers, e.g., corals and large benthic foraminifera (LBF), construct ecologically important habitats along the oceans tropical coasts and contribute considerably to global carbonate sediment production. Ocean warming is among the most damaging factors to coral reef ecosystems, often leading to the disruption of the photosymbiotic associations. Seen as bleaching, it may ultimately lead to mass mortality of reef-calcifiers. Understanding the characteristics that influence adaptive mechanisms of these photosymbiotic holobionts is hence crucial to project their future fate.

To disentangle the drivers of holobiont resilience, characteristics influencing the stress responses of both, host and photosynthesizing symbiont, need to be considered, including differences in species-specific adaptations and in local environmental conditions that may result in different acclimatization. This thesis aims to extend the understanding of adaptive mechanisms in photosymbiotic calcifiers in modern times of ocean warming by focusing on various levels of organismal responses of the common reef-associated diatom-bearing foraminifera *Amphistegina*, from populations to the proteomes of host and symbiont.

To test for inter-species and intra-species variations in thermal stress responses and symbiont assemblages, the widespread Indo-Pacific species *A. lessonii*, and its Atlantic counterpart *A. gibbosa*, were exposed to different ocean warming scenarios. Three thermal-stress treatments were simulated over one month in an experiment: a single thermal peak, followed by lower control temperature; episodic stress, simulated by four thermal peaks that alternated with periods at control temperature; and chronic stress. In addition to determining various parameters indicating holobiont and photosymbiont performance, the photosymbionts were characterized by genetic fingerprinting.

Although test populations of *A. gibbosa* were collected from habitats with different temperature ranges, their responses were similar, with only marginally higher tolerance to thermal peaks in specimens from a shallower-water site as compared to a deeper-water site in the direct vicinity. In contrast, differences between species bring evidence for higher tolerance of *A. lessonii* as compared to *A. gibbosa*, as episodic stress had no and chronic stress less pronounced impact, especially with regard to photosymbionts. These inter-species variations were consistent with the presence of different and more diverse symbiont assemblages in *A. lessonii*, which

demonstrates the importance of considering symbiont diversity in the assessment of stress response and adaptive capacity of LBF.

Monitoring performance of the deeper-dwelling group of *A. gibbosa* over the experimental period revealed that after three to twelve days, chronic stress led to bleaching, however, without inducing mortality, which may be a result of the steep increase in total antioxidant capacity in this treatment. Single and episodic stress induced both the same minor responses. As this population experiences fluctuating temperatures in its natural habitat, it is likely adapted to thermal peaks. This highlights the potential of such variable marine environments to support resilient physiological mechanisms among photosymbiotic organisms. Nonetheless, reproduction seemed to be suppressed by episodic and chronic stress. Such possible trade-offs may have far-reaching implications for LBF communities.

To reveal underlying molecular mechanisms, changes in the proteome were analyzed. A quantitative bottom-up proteomics approach was employed to link the cellular mechanisms to the observed stress responses in *A. gibbosa*. This offered the opportunity to separate the effect of the LBF host and its photosymbiont. High congruency to physiological parameters validated the presented novel workflow and showed major changes in the abundance of manifold proteins, induced by the different thermal-stress treatments. The proteome regulations going along with bleaching included the impairment of symbiont carbon concentrating mechanisms, and led to cell death and degradation. In the host, efficient repair mechanisms and enhanced protein synthesis maintained homeostasis, indicating higher thermal tolerance of the foraminifera compared to their symbionts. Metabolic pathways were adjusted to the symbiont loss, which demonstrates the importance of shifting feeding modes as resilience mechanism.

This thesis contributes to disentangling the underlying drivers of photosymbiotic reef organisms' responses to climate change, including the flexibility in symbiotic associations, interactions between host and symbionts, and the role of environmental factors shaping the range of their ecological constraints. The presented proteomics approach offers numerous possibilities to pinpoint cellular processes of the adaptive mechanisms among marine organisms.

Zusammenfassung

Fotosymbiose ist von essentieller Bedeutung für das Wohl und die Vermehrung vieler tropischer mariner Organismen, aber das Gleichgewicht innerhalb dieser Vergesellschaftung kann empfindlich auf Umweltveränderungen reagieren. Viele dieser riffbildenden Kalzifizierer, z.B. Korallen und Großforaminiferen, bauen ökologisch wichtige Habitate entlang der tropischen Meeresküsten auf und tragen wesentlich zu globalen Karbonatsedimenten bei. Ozeanerwärmung gehört zu den schädlichsten Einflüssen auf Korallenriffökosysteme, da sie oft zur Störung der fotosymbiotischen Gemeinschaft führt. Was zunächst als Bleiche sichtbar wird, kann letztlich zum Massensterben von Riffkalzifizieren führen. Es ist daher entscheidend, zu verstehen, wie die Anpassungsmechanismen dieser fotosymbiotischen Holobionten beeinflusst werden, um einzuschätzen, wie sie sich in Zukunft anpassen und entwickeln werden.

Um die Einflussfaktoren auf Resilienz von Holobionten zu verstehen, müssen alle Parameter, die das Stressverhalten von Wirt und fotosynthetisierende Symbionten beeinflussen können, verstanden werden. Dies umfasst auch die Betrachtung der artspezifischen Adaptionen und der lokalen Umweltbedingungen, die zu Akklimatisierung führen können. Mit der Betrachtung der verschiedenen organismischen Verhaltensebenen von Populationen zum Proteom von Wirt und Symbionten der weitverbreiteten riffassoziierten Foraminifere *Amphistegina* trägt die hier vorgelegte Dissertation zum Verständnis der Anpassungsmechanismen in fotosymbiotischen Kalzifizierern bei.

Die inner- und zwischenartlichen Variationen im Hitzestressverhalten und der Symbiontenvergesellschaftung wurden anhand von zwei Arten untersucht: der indopazifischen Art *A. lessonii* und der atlantischen *A. gibbosa*. Beide stammen aus ähnlichen Lebensräumen und wurden in einem einmonatigen Experiment unterschiedlichen Ozeanerwärmungsszenarien ausgesetzt. Drei Hitzestressszenarien wurden simuliert: ein einmaliges Hitzeereignis, gefolgt von niedrigerer Kontrolltemperatur; episodischer Stress, simuliert durch vier Hitzeereignisse, die sich mit Kontrolltemperaturperioden abwechselten; und schließlich chronischer Stress unter hoher Temperatur. Außer einer Reihe von Parametern, die die physiologische Leistungsfähigkeit der Holobionten und Symbionten anzeigen, wurden die Fotosymbionten durch genetische Fingerabdrücke charakterisiert.

A. gibbosa wurden in Lebensräumen unterschiedlicher Wassertiefe und somit mit unterschiedlichen Temperaturbereichen beprobt. Dennoch war ihr Verhalten im Experiment sehr ähnlich – die Exemplare aus dem flachermarinen Standort zeigten nur marginal höhere Toleranz gegenüber Hitzeereignissen. Im Gegensatz dazu zeigen zwischenartliche Differenzen, dass *A. lessonii* hitzetoleranter ist: episodischer

Stress hatte keine und chronischer Stress weniger ausgeprägte messbare Auswirkungen. Dieser Unterschied deckt sich mit der Anwesenheit anderer und diverserer Symbiontengesellschaften in *A. lessonii*, was die Relevanz der Symbiontenvielfalt bei der Bewertung von Stressverhalten und adaptiver Kapazitäten von Großforaminiferen aufzeigt.

Physiologisch reagieren die tiefermarinen Exemplare von *A. gibbosa* auf chronischen Stress nach drei bis zwölf Tagen mit Bleiche, die jedoch keine Mortalität zur Folge hatte. Dies könnte auf den steilen Anstieg der Antioxidantiengesamtkapazität in diesem Szenario zurückzuführen sein. Einzelne und episodische Hitzeereignisse riefen jeweils die selben schwachen Reaktionen hervor. Eine Erklärung ist, dass diese Population in ihrer natürlichen Umgebung stark fluktuierenden Temperaturen ausgesetzt und daher an Hitzeereignisse adaptiert ist. Lebensräume mit stark fluktuierenden Bedingungen könnten somit ein großes Potential haben, resiliente physiologische Mechanismen hervorzubringen. Dennoch schienen sowohl episodischer als auch chronischer Stress die Reproduktion zu erschweren. Derartige Effekte könnten weitreichende Implikationen für Foraminiferengemeinschaften haben.

Um grundlegende molekulare Mechanismen aufzudecken, wurden Veränderungen des Proteoms untersucht. Ein quantitativer Bottom-up-Proteomikansatz erlaubte es, die zellulären Mechanismen mit dem beobachteten Stressverhalten von *A. gibbosa* in Verbindung zu setzen. Dies ermöglichte es, die Reaktionen von Wirt und die Symbionten zu unterscheiden. Die hohe Übereinstimmung mit den gemessenen physiologischen Parametern belegte die Anwendbarkeit des Ansatzes. Bedeutende Veränderungen der Proteinabundanzen konnten auf die Reaktion auf die unterschiedlichen Hitzestressszenarien zurückgeführt werden. Zum Beispiel konnte gezeigt werden, dass mit der Bleiche eine Beeinträchtigung kohlenstoffkonzentrierender Mechanismen der Symbionten einhergehen, die zu Zelltod und Zellabbau führen. Der Wirt erhielt seine Homöostase durch effiziente Reparaturmechanismen und verstärkte Proteinsynthese, und adjustierte Stoffwechselwege an den Verlust seiner Symbionten. Dies zeigt eine höhere Hitzetoleranz der Foraminifere im Vergleich zu den Symbionten und betont die Bedeutung veränderlicher Ernährungsweisen für Resilienzmechanismen.

Die hier vorgelegte Dissertation soll zu einem grundlegenden Verständnis der Einflussfaktoren auf die Reaktion fotosymbiotischer Rifforganismen gegenüber Umweltveränderungen beitragen. Dies umfasst eine Betrachtung der Flexibilität symbiontischer Vergesellschaftungen, der Interaktionen zwischen Wirt und Symbionten, und der Rolle von lokalen Umweltbedingungen. Der hier erstmalig angewandte Proteomik-Ansatz eröffnet neue und vielversprechende Möglichkeiten, zelluläre Anpassungsmechanismen mariner Organismen funktional zu verstehen.

Conference Contributions

Outlined below are the abstracts of those conference and workshop contributions that are related to the work presented and discussed in the present thesis.

Stuhr M, Reymond CE, Sickmann A, Kucera M, Meyer A, Westphal H (CMA – *Computational Molecular Analysis Summer School 2014*, Wilhelmshaven, Germany)

(Reverse) Proteomics as tool for biodiversity research - Applications on Foraminifera

Without abstract: […] I am studying the phylogeny and resilience of the larger benthic foraminifera *Amphistegina* spp., representing sensitive indicators that react quickly to environmental change. Based on test shape alone, foraminifer identification largely disregards their high morphological plasticity. In most cases, plasticity is a direct and systematic consequence of environmental conditions as individuals react and develop in different ways in response to their environment, according to variations in their genome (structural differences in proteins or temporal and spatial protein expression patterns). Therefore, diversity on an intra-species molecular level is essential for the evolutionary potential and resilience of foraminifera. To establish if *Amphistegina* spp. are monophyletic along with their associated symbionts (i.e., analysis of cryptospecies), large-scale mass spectrometry based proteome analysis will be performed to reveal intra-species variations.

Stuhr M, Reymond CE, Kucera M, Westphal H (*GLOMAR PhD Days 2015*, Etelsen, Germany)

Reverse Proteomics as novel tool for biodiversity research on benthic Foraminifera

Ecosystems heavily rely on functional biodiversity within the system to build resilience to environmental variability. Foraminifera are a highly diverse group, constituting largely to sediments on coral reefs and playing important roles in biogeochemical cycles. Their fossil record provides major indicators for palaeo-ecological reconstructions. Larger benthic foraminifera harbour photosynthetic symbionts and, like corals, are particularly sensitive to environmental changes. Traditionally, foraminiferal species are identified by the morphology of their calcareous shell, which is a selective adaptation to environmental forces. This phenotypic plasticity is a consequence of the various levels with which individuals respond to environmental conditions, including morphological, physiological, behavioural or phenological traits. These are direct outcomes of structural differences in temporal and spatial protein expression. Amphistigenidae is one of the most recognised families of symbiont-bearing foraminifera, widely used in ecological studies. They react sensitively towards stress by test modification and

symbiont loss, but related processes and interactions with their photo-symbionts are widely unknown. Genetic analyses revealed numerous cryptic foraminifera species, following different evolutionary trajectories and distinctly responding to environmental forcing. Therefore, the proteomic responses of different populations of Amphistigenidae to chronic and repetitive environmental stress exposure will be characterized and linked to cellular biomarkers. Revealing diversity in Foraminifera on an intra-species molecular level is essential to understand their adaptive potential, evolutionary mechanisms and resilience towards environmental changes.

Stuhr M, Kucera M, Rieder V, Rahnenführer J, Westphal, H (*GLOMAR PhD Days 2015,* Etelsen, Germany)

Acclimatization of larger benthic foraminifera to long- and short-term thermal stress

Foraminifera are a highly diverse group, contributing strongly to calcium carbonate production within oceans and commonly used as bioindicators. Larger benthic foraminifera (LBF) harbor photosymbionts making them sensitive to environmental changes. Thermal stress represents one of the major threats to coral reefs worldwide, causing the loss of photosymbiotic algae we know as bleaching. Due to the projected increase in sea surface temperatures, photosymbiotic organisms are expected to be highly vulnerable. Therefore, it is important to understand how they will acclimate to elevated duration and frequency of thermal stress. To study the adaptive potential of LBF and identify underlying mechanisms, Amphistegina gibbosa populations were exposed to chronic, unique and repetitive heat stress over four weeks. Photosynthesis, coloration, and chlorophyll a content were measured to determine the symbiotic algae response in a temporal resolution, while growth, respiration, and cellular biomarkers were measured regularly to understand the holobionts response. Under chronic stress, a bleaching process was observed, accompanied by lower photosynthesis rates. This was reflected in decreased motility, growth, and an increase in the antioxidant capacity against peroxyl radicals, thus an immune response. The short-term stress in contrast did not show such significant effects, but rather firstly enhanced activity. Linking these responses indicates changes in the symbiotic relationship between foraminifera and algal endosymbionts.

Stuhr M, Reymond CE, Kucera M, Westphal H (*GSA – Geological Society of America, Annual Meeting 2015*, Baltimore, USA)

Acclimatization potential of *Amphistegina* spp. and their symbionts to long- and short-term thermal stress

Larger benthic foraminifers (LBF) are widely used to reconstruct modern and ancient marine environments due to their high diversity and specific niche

requirements. This has made them a widely used bioindicator within coral reef ecosystems with which to assess ecosystem health. One of the major threats to coral reefs worldwide is thermal stress, which causes the loss of photosymbiotic algae, more commonly know as bleaching. Due to the projected future increase in sea surface temperatures (SST) photosymbiotic organisms are expected to be highly vulnerable. This phenomenon is more commonly known for corals, however it has been observed among LBF since the 1990's. Therefore, it is important to understand how photosymbiotic marine organisms will adapt to an elevated intensity, duration, and frequency of thermal stress. To study the adaptation potential of photosymbiont bearing benthic foraminifera towards thermal stress and to identify the underlying mechanisms of acclimatization and recovery, experimental studies were conducted on the genus *Amphistegina* to assess chronic, unique and repetitive heat stress over four weeks. To monitor intra- and interspecific variations three populations from two different geographic regions (Florida Keys and Zanzibar) and depths (5 m and 20 m) were used. Specifically, growth, respiration, and cellular biomarkers (such as total antioxidant capacity against peroxyl radicals) were measured to understand the holobiont response, while photosynthesis, coloration, and chlorophyll a content were measured to determine the photosymbiotic algae response. Variations in these parameters indicate changes in symbiont biomass and fitness as well as enzymatic activities of the foraminiferal antioxidant defense system. These findings show alterations in the symbiotic relationship, such as bleaching, acclimatization and possible higher resilience to various combinations of stress events. Understanding these intra- and interspecific variations and acclimatization mechanisms is key to deciphering the range of responses in marine photosymbiotic organisms to global warming.

Blank-Landeshammer B, Biß K, Kollipara L, Rieder V, **Stuhr M,** Schell T, Zahedi R, Pfenninger M, Rahnenführer J, Westphal H, Sickmann A (*ASMS – American Society for Mass Spectrometry, Annual Conference 2016*, San Antonio, USA)

Novel approaches in *de novo* peptide sequencing and proteogenomics as tools to explore uncharted organisms

The diversity of species and their ecosystems are contributing importantly to our major sources of food and medicine, building the basis of our daily life. Biodiversity degradation due to excessive human impact is currently a major concern and suitable taxonomic classification is often amiss. For identification, DNA barcoding is available, but with certain limitations. Therefore, proteomics-based profiling of distinct organisms to identify species-specific proteome signatures could alternatively provide valuable information, even for species developing quickly under environmental pressure. This data should be complementary to those species with available genomic data, whereas for non-model organisms with unsequenced genomes, it could emerge as a unique way of taxonomic classification. In this work,

Radix spp. (Phylum: Mollusca; common pond snail) and *Amphistegina* spp., (Phylum: Foraminifera; unicellular eukaryote) were selected as model organisms. Raw genome data is available for *Radix* spp., whereas no genomic data for the genus *Amphistegina* spp. is accessible yet. Sample preparation for LC-MS/MS analysis was carried out by typical bottom-up proteomics workflow involving lysis, carbamidomethylation and on-filter enzymatic proteolytic digestion. The annotation of the generated MS/MS data was done by multiple *de novo* peptide sequencing algorithms such as, PEAKS, RapidNovor and pNovo+. Furthermore, trypsin mediated $H_2{}^{18}O$ labeling was used to facilitate unambiguous y-ion identification. Lastly, a statistical algorithm to determine the similarity of taxa based on their MS/MS peptide spectra has been developed. We used three different approaches to validate the potential of mass spectrometry-based proteomics for biodiversity research. Firstly, through optimization of MS data acquisition settings, quality and accuracy of *de novo* peptide sequencing results could be significantly increased, leading to the identification of approx. 12,000 peptide sequences per foraminifera-species and more than 50,000 in *Radix auricularia*. Overlaps between technical replicates were ranging between 55 and 60%. Validation with complex samples of known composition enabled the estimation of false-discovery rates using a novel search strategy. Thus, the annotation rate of MS/MS spectra could be increased up to four-fold while maintaining an estimated FDR value below 5%. Homology searches of the generated high-confidence peptides enabled the differentiation of foraminiferal peptides and ones originating from symbiotic algae. Secondly, to identify potential translational start sites in the raw genome of *Radix auricularia*, we aimed to identify potential protein N-termini. Therefore, we conducted N-terminal peptide enrichment via charge based fractional diagonal chromatography (ChaFRADIC), followed by LC-MS analysis. Combination of *de novo* peptide sequencing with proteogenomic workflows led to further improvement of novel gene annotations not covered by *ab initio* gene prediction software. Thirdly, by the use of a new algorithm comparing the similarity between pairwise MS/MS datasets, it was possible to resolve taxonomy-level differences of samples based on the MS/MS spectra generated from 2h LC/MS measurements - without the need of further spectral identifications.

Stuhr M, Reymond CE, Kucera M, Blank-Landeshammer B, Kollipara L, Rieder V, Rahnenführer J, Sickmann A, Westphal H (*ICRS – 13th International Coral Reef Symposium 2016*, Honolulu, USA)

Application of mass spectrometry-based proteomics to study large benthic foraminifera and their molecular responses to environmental changes

A new method for studying larger benthic foraminifera (LBF) using novel proteomic techniques was developed. Foraminifera are a highly diverse group, contributing strongly to calcium carbonate production within oceans and commonly used as

environmental indicators. LBF harbor photo-symbionts making them vulnerable to environmental changes, especially ocean warming. Due to their small size, morphological identification, genetic and traditional protein studies are challenging. Even for the cosmopolitan group Amphisteginidae, very little is known about their molecular biology. Therefore, cellular processes occurring in response to environmental changes are widely unknown. The presented method is based on the complete proteome analysis by liquid chromatography-mass spectrometry (LC-MS). This method allows differentiation between taxa by identification of tandem mass spectra via *de novo* peptide sequencing and homology searching as well as a new approach comparing unidentified mass spectra by computing the similarity between datasets. Moreover, label-free quantification of peptides detects changes in the relative abundance of certain protein-candidates in response to stress. Results from a temperature experiment on *Amphistegina gibbosa* show variations in the quantity of peptides linked to physiological stress. The presented approach gives deeper insight into the compartment specific molecular responses, induced in the foraminiferal host or photo-symbionts, offering new methods to monitor stress responses in this host-symbiont system.

Stuhr M, Reymond CE, Kucera M, Blank-Landeshammer B, Kollipara L, Sickmann A, Westphal H (*Batsheva de Rothschild Workshop 2016: Live foraminifera as a new model system*, Eilat, Israel)

Understanding the molecular basis for stress response in foraminifera and symbionts by proteome analysis.

The proliferation of calcification by coral reef building organisms greatly relies on the mutual association with photosymbiotic algae, albeit making the holobiont more sensitive to light and temperature fluctuation. To evaluate the adaptive capacity of photosymbiont-bearing reef organisms such as large benthic foraminifera (LBF), it is essential to understand the underlying stress response mechanisms at the cellular level. To date, probing proteins in host and symbiont cells and partitioning both components are major obstacles for ecological studies. Recent advances in protein analysis in foraminifera e.g., expression of RuBisCO and Hsp70 provide new physiological insights, but these low- to medium-throughput analysis are elaborate and target only single proteins. To overcome these limitations, we applied bottom-up proteomics workflow and mass spectrometry-based label free quantification to LBF holobionts. Changes in relative abundances of proteins in response to different environmental stress conditions are thereby determined. Protein identification by database and homology searching allows for the partial assignment of proteins either to the host or the photosymbionts. In a temperature stress experiment on *Amphistegina gibbosa*, we quantified 6,000 peptides, which are associated to 1,600 proteins of phylogenetically related species (≥1 unique peptide; 1% false discovery rate). Among the symbionts,

photosynthesis-related proteins were significantly reduced, while higher abundance of stress response proteins such as Hsp70 indicates thermal stress. In the foraminiferal host, high water temperatures relatively increased proteins involved in metabolic pathways and in building the cytoskeleton. These changes were reflected in measured physiological parameters e.g., oxygen production, which demonstrates how thermal stress impacts the protein expression of symbionts and related shifts in the hosts metabolism. The presented proteomics approach offers new opportunities to study component specific molecular responses in LBF and provides novel insights into their adaptive mechanisms towards environmental changes, especially ocean warming.

Kollipara L, Blank-Landeshammer B, Biß K, Rieder V, **Stuhr M,** Schell T, Zahedi R, Pfenninger M, Rahnenführer J, Westphal H, Sickmann A (*Proteomic Forum 2017*, Potsdam, Germany)

Biodiversity research based on a combinatorial approach comprising proteomics, proteogenomics and MS/MS spectral similarity

Declination of biodiversity due to excessive human intervention is currently a major concern and suitable taxonomic classification is often amiss. Classification of species enables to clearly identify, study and to target conservation efforts. Conventional species identification technique i.e., DNA barcoding, although rapid, is prone to errors. Proteomics-based profiling of distinct organisms to identify species-specific proteome signatures in conjunction with proteogenomics and MS/MS spectral similarity approaches could provide valuable information. Here, *Radix* spp. (Phylum: Mollusca; common pond snail) and *Amphistegina* spp., (Phylum: Foraminifera; unicellular eukaryote) were selected as model organisms. Raw genome data is available for *Radix* spp., whereas no genomic data for *Amphistegina* spp., is accessible yet. Sample preparation for LC-MS/MS analysis was carried out by typical bottom-up proteomics workflows (e.g., FASP) and the annotation of the generated MS/MS spectra was done by a combination of database searching and multiple *de novo* peptide sequencing algorithms. By optimization of MS data acquisition settings, quality and accuracy of *de novo* peptide sequencing ~12,000 peptide sequences were annotated per foraminifera-species. Homology searches of the generated high-confidence peptides enabled the differentiation of foraminiferal hosts and their symbionts. Moreover, to refine *ab initio* gene prediction, a dual-approach was developed, comprising *de novo* peptide sequencing and a reductive database search. Here, spectra not matching in a first search against predicted protein sequences are subjected to a two-step search against the whole genome. Thus, 50% of the predicted protein sequences in *R. auricularia* could be confidently confirmed by 184,689 peptides (1% FDR on protein level), with 56,520 additional peptides not covered by gene prediction. Lastly, by implementing a novel algorithm for comparing the similarity between pairwise MS/MS datasets enabled to resolve

taxonomy-level differences of samples without the need for database searches. In conclusion our approach comprising proteomics, proteogenomics and spectral matching platforms can facilitate and speed up the taxonomic species classification.

Stuhr M, Blank-Landeshammer B, Reymond CE, Sickmann A, Ries JB, Westphal H (*52nd European Marine Biology Symposium 2017*, Piran, Slovenia)

Proteomic response of photosymbiont-bearing foraminifera to global impacts on ocean conditions

The proliferation of coral reef building organisms greatly relies on the mutual association with photo-symbiotic algae, albeit making the holobiont more sensitive to light and temperature fluctuation. A key ecological engineer and highly diverse group of (sub-) tropical carbonate producers harboring algal endosymbionts are large benthic foraminifera (LBF). They are widely used as bioindicators of ecosystem health and, much like corals, they are vulnerable to projected environmental changes. With respect to of global impacts such as ocean warming and acidification, acclimatization and adaptive physiology are highly important to the future of photosymbiotic reef calcifiers. To evaluate their adaptive capacity, it is essential to understand the underlying mechanisms involved in stress responses at the cellular and molecular level. To date, probing proteins in host and symbiont cells and partitioning these two components are major obstacles for ecological studies. Even for the cosmopolitan group Amphisteginidae, very little is known about their molecular biology. Therefore, we applied a label-free shotgun proteomics approach to detect changes in the relative abundance of protein-candidates in response to environmental stress and subsequent protein identification allowed for the partial assignment of proteins to either the foraminiferal host or the endosymbiotic algae. In a combined 2-months ocean acidification and temperature stress experiment on *Amphistegina lobifera*, a common LBF currently invading the Mediterranean, we universally identified and relatively quantified >1,000 protein cluster. Elevated temperature (28°C) was found to have stronger influence on the LBF than even extremely high seawater pCO_2. At ambient temperature (25°C), higher pCO_2 appears to fertilize the photosymbionts and thereby increase holobiont growth. The interaction of both variables, ocean warming and acidification, did not induce a linear response, but indicated a highly complex response system. Under thermal stress only, photosynthesis-related proteins were reduced among the symbionts, while stress response proteins and chaperones increased in abundance. In the foraminiferal host, metabolic pathways seemed to be affected by allocation of energy resources. By allowing component partitioning, proteomic approaches provides novel insights into the cellular response mechanisms of LBF towards environmental changes and offer opportunities to monitor interactions in this host-photosymbiont system.

Overview of Publications and Manuscripts

The presented dissertation is a cumulative doctoral thesis that consists of one paper published in an international peer-reviewed journal (chapter 3) and two submitted manuscripts that are currently under review (chapters 2 and 4).

These chapters are framed by a general introduction including a description of the methodological approach and outline (chapter 1), an extended discussion (chapter 5) and recommendations for future research (chapter 6). The contributions of the authors to the manuscripts are summarized on the backside of each respective cover page.

Chapter 2: **Stuhr M**, Meyer A, Reymond CE, Narayan GR, Rieder V, Rahnenführer J, Kucera M, Westphal H, Hallock P (under review) Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera *Amphistegina* linked to differences in symbiont type. Under review at *Coral Reefs*

Chapter 3: **Stuhr M**, Remond CE, Rieder V, Hallock P, Rahnenführer J, Westphal H, Kucera M (2017) Reef calcifiers are adapted to episodic heat stress but vulnerable to sustained warming. *PLoS ONE 12(7): e0179753*

Chapter 4: **Stuhr M**, Blank-Landeshammer B, Reymond CE, Kollipara L, Sickmann A, Kucera M, Westphal H (under review) Disentangling thermal stress responses in reef-calcifiers and their photosymbionts by shotgun proteomics. Under review at *Scientific Reports*

In addition, the research project resulted in a further methodological manuscript, co-authored by the candidate, which is cited in the thesis, but does not constitute a chapter:

Rieder V, Blank-Landeshammer B, **Stuhr M**, Schell T, Biß K, Kollipara L, Meyer A, Pfenninger M, Westphal H, Sickmann A, Rahnenführer J (2017) DISMS2: A flexible algorithm for direct proteome-wide distance calculation of LC-MS/MS runs. *BMC Bioinformatics 18:148*

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Chapter Six

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communication was sometimes challenging, however, I feel like we turned this obstacle into a source of professional and personal advance, which was one of the most precious and pleasing lessons to me. Hence at ISAS, I thank Karsten for first introducing me patiently into the art of proteome analysis, and Bernhard, Lucky and Ingo for providing me with great support in analyzing samples and huge data sets, but also for the amusing excursions during my stays in Dortmund. I am also grateful to Vera and Jörg from the TU Dortmund for their professional advice and extensive assistance in statistical data analysis.

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An understanding of the natural world and what's in it is a source of not only a great curiosity but great fulfillment.
– David Attenborough –

Chapter One
GENERAL INTRODUCTION

1.1 Coral reef organisms during times of climate change

The rapid rate of climate change, resulting from anthropogenic greenhouse gas emissions, leads to alterations of the chemical and physical conditions in marine ecosystems (IPCC 2013; Hoegh-Guldberg et al. 2017). Among the various effects of global change, the rise in seawater temperatures, here referred to as ocean warming (OW), is currently perceived to be the major threat to coral reef ecosystems (Hughes et al. 2017). Average sea surface temperatures (SST) in warm-water coral-reef regions have risen by 0.44 to 0.79°C from 1950 to 2009 (Hoegh-Guldberg et al. 2017) and are predicted to further increase to 2.6 to 4.8°C (with a mean increase of 3.7°C) until the end of the century (Representative Concentration Pathway [RCP] 8.5; IPCC 2013). Such warming will induce physiological stress on many marine species, resulting in loss of fitness and functional impairment. The most prominent example is the currently observed decline in the health of reef-building corals, which is in contrast to their stability during the past millennia (Hughes et al. 2003, 2017; Pandolfi et al. 2003; Hoegh-Guldberg et al. 2007, 2017). Coral reefs, the 'rainforests of the sea', are hotspots of biodiversity and count among the most productive ecosystems on earth (Odum and Odum 1955; Burke et al. 2011). Their degradation will lead to the loss of their ecosystem services, including carbonate production, and habitat construction, ultimately affecting the livelihood of hundreds of millions of people (Moberg and Folke 1999; Burke et al. 2011).

1.1.1 Ocean warming and holobiont bleaching

The adverse effects of OW on coral reef ecosystems result from disruptions in the sensitive relationship of many reef calcifiers with their photosynthesizing endosymbiotic microalgae. In corals, thermal stress leads to increased production of reactive oxygen species by the endosymbiotic dinoflagellate *Symbiodinium*. The induced oxidative stress damages the coral host (Lesser 2006) and the photosymbionts and/or -pigments are expelled during heating events. This phenomenon is known as coral bleaching. Although it is partly reversible, in the long-term, it may lead to extensive mass mortality (Glynn 1996; Baker et al. 2008). With ongoing OW, coral reef ecosystems are exposed to weather anomalies and the related temperature stress with increasing frequency, and the recovery time between stress events for these organisms is narrowing (Donner et al. 2005). For the Florida Keys, yearly bleaching events are predicted to occur between 2020 and 2045 (Manzello 2015), while the majority of reefs worldwide are expected to bleach annually by mid-century under an OW projection based on the business-as-usual emission scenario RCP8.5 (IPCC 2013; van Hooidonk et al. 2014). Besides corals, other photosymbiotic reef calcifiers, such as large benthic foraminifera (LBF), calcifying algae or giant clams are equally affected by climate change and were also found to bleach in response to thermal stress (Talge & Hallock 2003; Addessi 2001; Anthony et al. 2008; Schmidt et al. 2011).

1.1.2 Reef-associated foraminifera

Foraminifera are extensively studied by geologists for their spectacular fossil record and their biostratigraphic value, but they also constitute one of the most diverse and abundant groups of calcifying organisms in modern marine environments (Sen Gupta 2003). This is because most of these unicellular eukaryotes construct external shells (*tests*) that encompasses the cell and contribute importantly to the flux of biogenic carbonate in the world oceans (Langer et al. 1997; Langer 2008). The tests have one or more openings (apertures) from where they extend granuloreticulopodia to collect food, move, attach themselves to substrate, and induce chamber formation (Fig. 1-1a–c) (Lee and Anderson 1991).

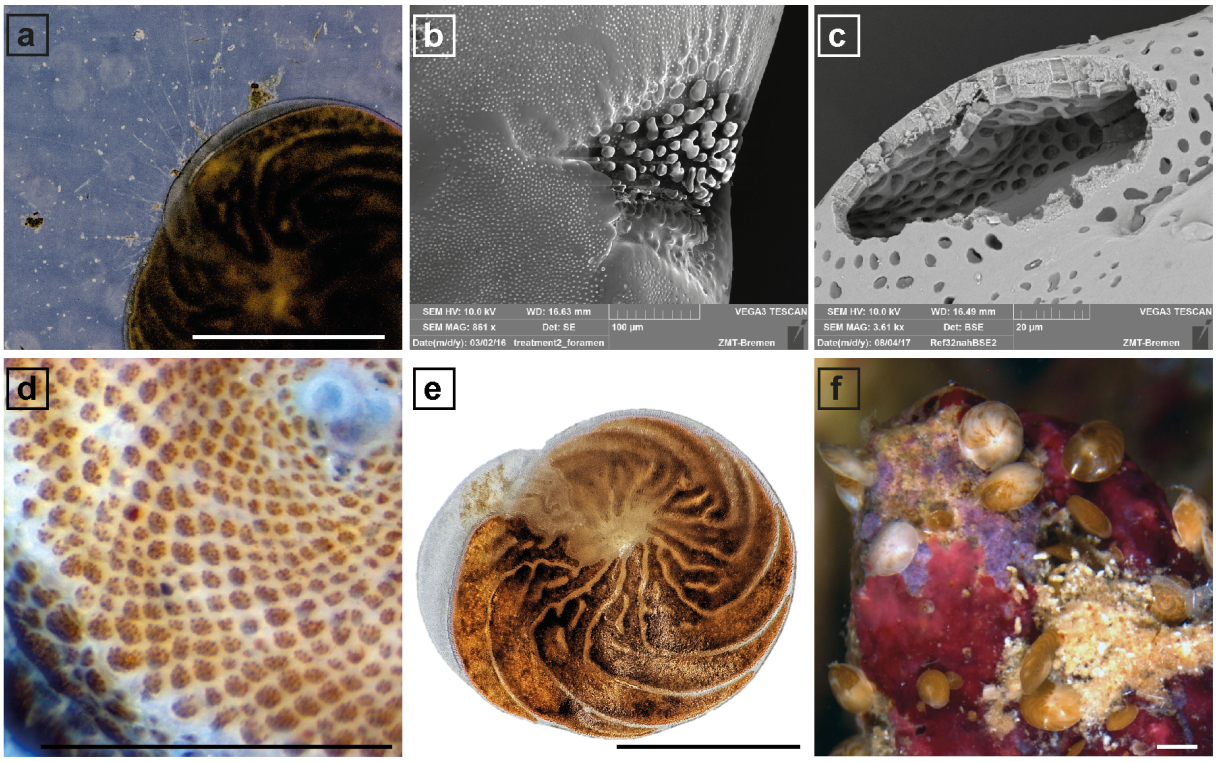

Figure 1-1. Features of (photosymbiotic) foraminifera: a) high definition range (HDR) image of Amphistegina lessonii *extending its granuloreticulopodia (bright lines spreading from the test) to attach and move itself, and to collect food particles from the surroundings; b) scanning electron microscopy (SEM) image of the aperture of* A. gibbosa *seen from the ventral side; c) SEM image of broken chamber of* A. lobifera *showing the pores typical for perforate foraminifera and the layers of calcite building the skeleton; d) close-up of the imperforate* Amphisorus *sp. showing the individual dinoflagellate symbionts located in the foraminiferal chambers; e) HDR image of* A. lessonii *showing green-brown coloration of symbionts that are moved into the outermost newly-build chamber; f)* A. gibbosa *in culture showing different extents of bleaching, ranging from healthy golden-brown specimens to slight and intermediate signs of bleaching, seen as white spots and 'mottling', to severe symbiont loss in nearly entirely white individuals; all scale bars: 0.5 mm.*

By far the greatest proportion of foraminiferal taxa live in benthic marine habitats. Many species living in shallow areas of the warm (sub-) tropical realm live in symbiosis with various types of endosymbionts (Fig. 1-1d–f). Because this way of life evolved multiple times in different groups of foraminifera and allows them to grow to great sizes (Lee and Hallock 1987; Stanley Jr and Lipps 2011), members of this ecological group are vernacularly referred to as large benthic foraminifera (LBF) (Fig. 1-2). The most common endosymbiont of LBF are diatoms, but symbioses with dinoflagellates, rhodophytes, green algae and cyanobacteria have also been identified in the group (Lee 2006) and the first likely evidence of LBF photosymbiosis can be traced back to the extremely abundant Paleozoic fusulinids (Lee and Hallock 1987). While the association between foraminiferal families to certain types of symbionts seems to be finical (i.e., a LBF family harbors either diatoms or dinoflagellates, but never both), the flexibility to associate with different genera or species of that symbiont type is thought to be rather high (Lee et al. 1997).

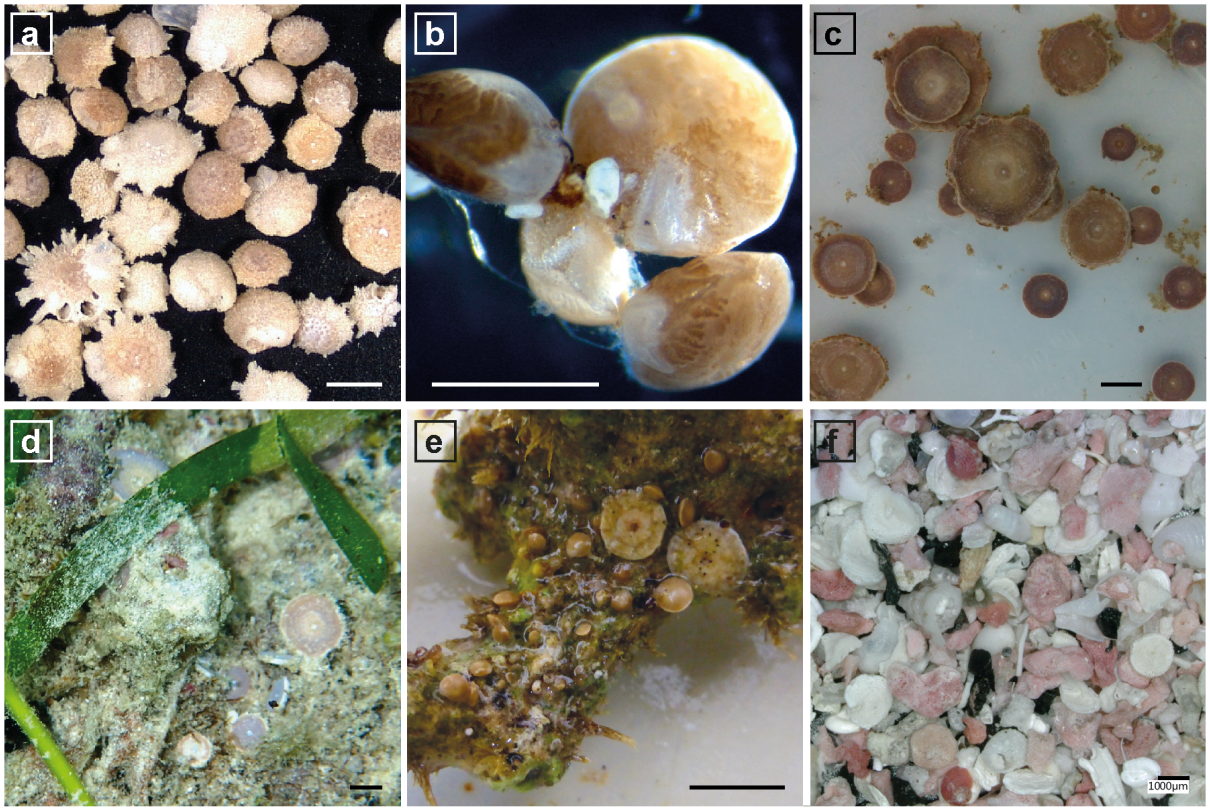

Figure 1-2. Common large benthic foraminifera that may contribute substantially to the production of marine carbonates, e.g., a) the diatom-bearing Calcarina *sp. from Koh Pha Ngan, Gulf of Thailand and b)* Amphistegina lobifera *from Crete, Mediterranean, and c) the dinoflagellate-bearing* Marginopora vertebralis *from Zanzibar, western Indian Ocean, d) where it occurs in high quantities in the local seagrass patches, e) while* Amphistegina *and* Sorites *spp. are found numerous on hard carbonate substrates such as coral rubble, Eilat, northern Red Sea; all scale bars: 0.5 mm. f) high abundance of LBF in the local habitats creates sediments rich in foraminiferal tests, Crete, Greece; scale bar: 1 mm.*

The energetic advantages of algal symbiosis such as the provision of additional carbohydrates released from the symbionts to the host (Lee et al. 1984; Hallock 2000) as well as enhancement of calcification due to lowered alkalinity (Nooijer et al. 2009) allows LBF to substantially contribute to the production of carbonate sands in coral reefs (Baccaert 1986; Langer 2008; Doo et al. 2012a), locally generating >75% of the sediment (Hohenegger 2006; Dawson and Smithers 2014). Thereby, they act as ecosystem engineers and aid in stabilizing sand cays and reef structures (Yamano et al. 2000; Fujita et al. 2009; Langer et al. 2012; Weinmann et al. 2013). Moreover, the post-mortem dissolution of their calcite test during nighttime can buffer daily pH fluctuations in shallow reef habitats (Yamamoto et al. 2012).

Due to their short life cycle and delicate photosymbiosis, LBF react quickly to environmental change, which makes them sensitive bioindicators (Hallock et al. 2003). They therefore provide valuable proxies for the reconstruction of past environmental conditions as well as tools for the assessment of ecosystem health status in recent marine habitats (Cockey et al. 1996; Hallock et al. 2003). Because they are relatively small and abundant, collection and cultivation of statistically significant sample sizes is fast and cheap with a minimal impact on local reef resources (Fig. 1-3). As photosymbiotic calcifiers, they provide an analog to tropical corals and an exceptional model to conduct experiments and surveys testing the responses of environmental impacts on photosymbiotic reef calcifiers (Lee and Anderson 1991; Prazeres et al. 2017b).

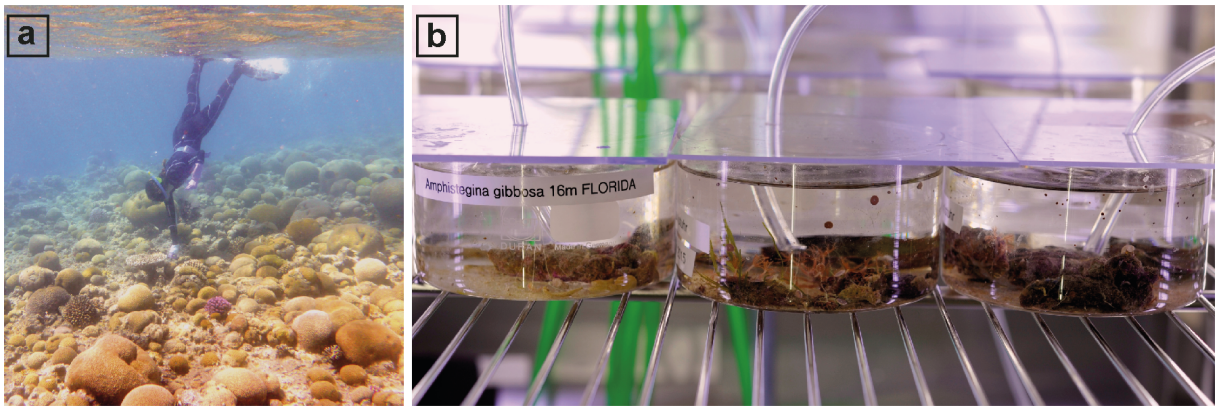

Figure 1-3. Foraminifera sampling and cultivation: a) collection of coral rubble can be obtained with relatively little logistic efforts, even by snorkeling in very shallow reefs such as in Eilat, northern Red Sea; b) foraminifera can be easily cultured over several years in glass dishes bubbled with air in climate chambers with controlled light and temperature levels, such as in the marine experimental facility MAREE at ZMT.

1.1.3 Impacts of ocean warming on photosymbiotic foraminifera

Many studies in recent years have highlighted the susceptibility of LBF to the impacts of climate change. OW and high light intensities are known to cause bleaching in LBF (Talge and Hallock 2003; Schmidt et al. 2011), which is manifested as a reduction in photosymbiont density and performance, and impacts the holobiont respiration, motility, growth, survivorship and fecundity (Hallock et al. 2006; Schmidt et al. 2011, 2014; Doo et al. 2014a; Prazeres and Pandolfi 2016; Prazeres et al. 2016b). These impacts are often accompanied by increased proportions of malformed or broken tests, infections and reproductive dysfunction, including partial or complete failure of asexual reproduction and abnormal offspring morphologies (Hallock et al. 1995; Talge and Hallock 1995; Toler and Hallock 1998; Hallock 2000). Histological studies of LBF bleached by exposure to high light conditions indicate that the process starts with the deterioration of the symbiont chloroplasts, followed by subsequent digestion of the symbionts by the foraminifera, and ultimately autolysis of the hosts cytoplasm (Talge and Hallock 1995). Recent studies using protein-expression analysis and cellular biomarkers indicate that the photosynthetic carbon-fixation enzyme Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO) as well as holobiont antioxidant capacity, Mg-ATPase and Ca-ATPase activity are negatively impacted by thermal stress (Doo et al. 2012b; Prazeres and Pandolfi 2016; Prazeres et al. 2016b) while foraminiferal heat shock proteins are expressed increasingly (Heinz et al. 2012). Beyond these single-marker studies, the cellular mechanisms of LBF bleaching (Fig. 1-1f) in response to OW remain poorly known. In a symbiotic association, one compartment (symbionts or host) may react differently to changing environmental conditions than the other. Therefore, understanding the impacts of global change stress on these organisms requires a deconvolution of the response mechanisms to thermal stress between both compartments.

1.2 Adaptive mechanisms of photosymbiotic reef organisms

In the face of severe environmental changes, the future of coral reef organisms is likely dependent on their resilience (Hughes et al. 2005), i.e., the ability to resist change and capacity to recover, or capacity to adapt rapidly. It has been recently shown that acclimatization via phenotypic plasticity can protect coral populations during rapid environmental changes (Pandolfi et al. 2011; Mayfield et al. 2013; Munday et al. 2013). Assuming that high phenotypic plasticity correlates with high genotypic plasticity and evolvability, this mechanism may facilitate their survival under ongoing warming (Palumbi et al. 2014; Torda et al. 2017). Therefore, to predict the fate of photosymbiotic reef organisms, we need to take phenotypic / physiological plasticity into account and uncover the causes that determine their susceptibility to different stressors. In holobionts such as LBF and corals, i.e., partnerships between hosts and their resident photosynthesizing algae, the ways in which individual traits contribute to stress response (Fig. 1-4), in combination or

isolated, are particularly complex and numerous (Weis 2010). Determining the susceptibility / resilience of a photosymbiotic holobiont and the likelihood to alter this state with climate change is hence a challenging endeavor that needs to elucidate multiple factors and mechanisms.

1.2.1 Acclimatization and adaptation

Changes in holobiont resilience can either be caused by genotypic adaptation, i.e., natural selection driven by differences in susceptibility over evolutionary time scales, or by phenotypic acclimatization, i.e., the individuals' response to extremes based on its existing genetic repertoire within its lifespan (Weis 2010). In corals, host genotypic adaptation to different thermal regimes has been observed (Barshis et al. 2010), as well as phenotypic plasticity of both host- and symbiont-specific traits in response to thermal stress (Baker 2003). In planktonic foraminifera, molecular analyses showed that some morphospecies actually include several genotypes (Kucera and Darling 2002), which implies that these distinct genetic entities have different biogeographies and ecologies (Darling and Wade 2008). Genotypic distinction in LBF hosts has been detected over large latitudinal gradients (Garcia-Cuetos et al. 2005; Lee et al. 2016; Schmidt et al. 2016a), but these do not necessarily represent thermal adaptations. So far, molecular studies differentiating genotypes in some LBF genera such as *Amphistegina* are still obstructed by difficulties developing unique primers to sequence their unusual and variable DNA such that genotypic differences and phylogenies of these LBF remain unresolved. Anyhow, variations in thermal thresholds of LBF morphospecies (herein generally referred to as species) have been described (Schmidt et al. 2011; Engel et al. 2015; Titelboim et al. 2016), but influence of the other characteristics mentioned below cannot be excluded by the given study designs and further research is hence awaiting.

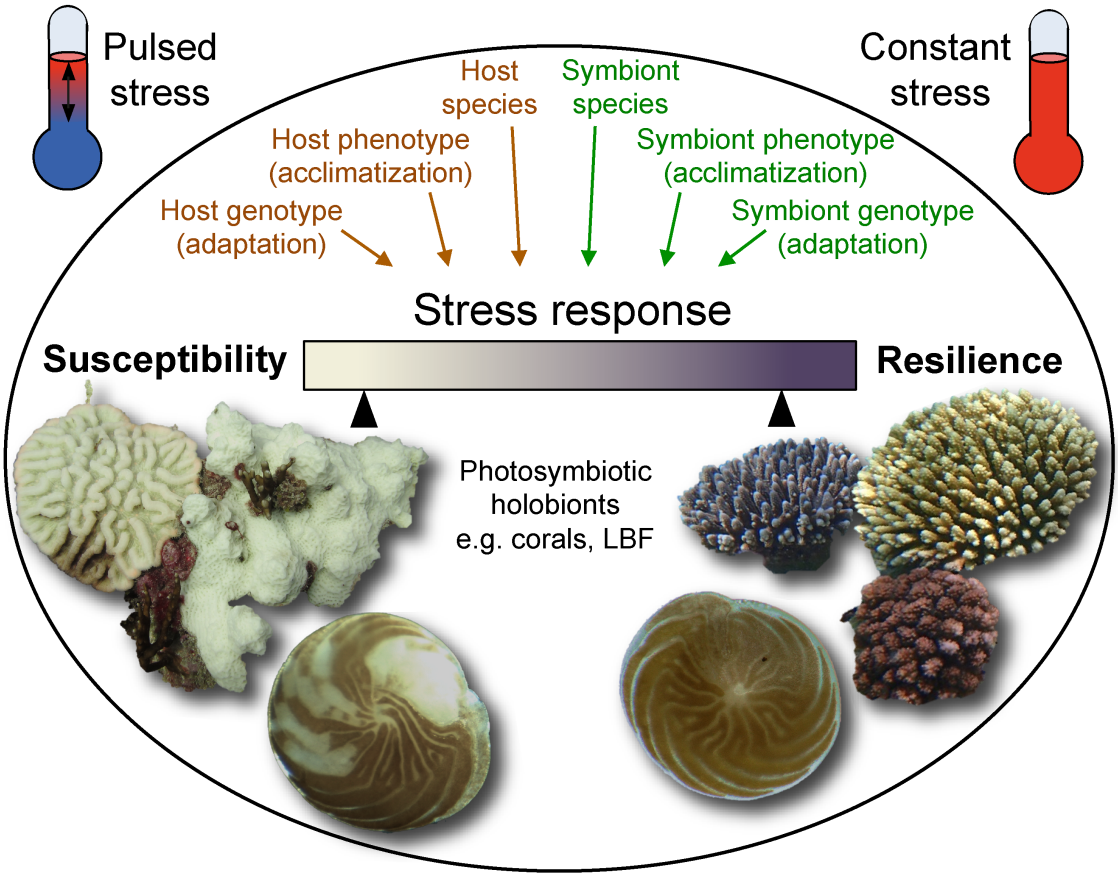

Figure 1-4. Characteristics of photosymbiotic holobionts that could contribute to stress responses and adaptive mechanisms. Acting alone in or in combination, these factors result in a response that falls along a continuum from susceptibility to resilience. Highly susceptible corals or large benthic foraminifera (LBF) often bleach whereas resilient ones keep their symbionts (modified after Weis 2010). These responses may further depend on the character of the stressor, e.g., thermal stress, can be expressed as constant warming or as strong fluctuations that create thermal peaks.

1.2.2 The role of the symbionts in thermal stress response

The characteristic that has caught most attention among coral ecologists is the relationship between different host and symbiont species and their combinations. A possible mechanisms of thermal acclimatization observed in corals includes the shift to more thermally tolerant symbiont lineages after a bleaching event (Thornhill et al. 2006), referred to as symbiont shuffling. Moreover, the partnerships between corals and their dinoflagellate symbionts show varying degrees of specificity/flexibility, which may, in their diverse nature, significantly influence the resilience of coral reefs (Baker 2003). The specificities of coral hosts to certain symbiont clades and the different thermal adaptations of various *Symbiodinium* lineages have been studied extensively (e.g., Macdonald et al. 2008; Barshis et al. 2010; Oliver and Palumbi 2011; Grégoire et al. 2017). For most other photosymbiotic-calcifiers such as LBF, such knowledge is scarce (Lee 2006; Lee et al. 2010). Experiments that cultured endosymbionts from LBF indicate the existence of up to ~20 different species of potentially symbiotic diatoms, with *Nitzschia frustulum* var. *symbiotica*, *Nitzschia panduriformis* var. *continua*, *Nitzschia laevis*, *Nanofrustulum shiloi*, *Amphora*

tenerrima and *Amphora roettgerii* representing 75% of all isolations, but no preferred relationship between hosts and symbiont species appears to be found (Lee and Correira 2005). Mostly, only one species of endosymbiotic diatom per specimen was found, but sometimes two or three species have been detected simultaneously (Lee 2006). A high diversity of diatom symbionts is presumably supporting the adaptation of LBF to a wide range of different light habitats and thereby explains their broad ecological range of occurrence as well as their high abundances in varying reef habitats (Nobes et al. 2008). Conversely, early cytological *in situ* analyses of diatoms within their foraminiferal hosts (Leutenegger 1983, 1984) and recent studies based on genetic identification of diatom symbionts (Holzmann et al. 2006; Schmidt et al. 2015) suggest that the symbiosis between LBF and diatoms may not be as loose as assumed (Barnes 2016). Up to now, it is not sufficiently known how variable the symbiont community is on temporal or seasonal scales, spatial over populations and across biogeographic or depth-related environmental gradients as well as between and within foraminiferal species (Lee et al. 2010).

1.2.3 The role of thermal fluctuations in reef environments

Recent studies suggest that organisms that experience greater environmental heterogeneity in their natural habitat have higher phenotypic plasticity and are thus more resilient to disturbances. For example, corals from habitats with high thermal variability or other environmental stressors have been found to be less prone to bleaching (McClanahan et al. 2007; Guest et al. 2016) than corals living in intertidal rock pools (Oliver and Palumbi 2011b; Smit and Glassom 2017) or reefs naturally exposed to large amplitude internal waves (Soto et al. 2011; Schmidt et al. 2016c). The thermal history of coral reef organisms is accordingly an important factor that may increase its acclimatory responses (Middlebrook et al. 2008; Ruiz-Jones and Palumbi 2017). Besides their function in increasing resilience mechanisms, temperature fluctuations, e.g., induced by storms, large amplitude internal waves or local upwelling (Mayfield et al. 2013; Buerger et al. 2015) may also reduce or temporarily disrupt thermal stress in coral reef habitats, alleviating its detrimental effects by allowing for short-term recovery of the local community (Wall et al. 2015).

OW is not only expressed through the increase in mean ocean temperature, but mostly occurs in transient local heating events of variable time scales (Leichter et al. 1996; IPCC 2013). Therefore, static temperature conditions often applied in thermal stress experiments do not realistically reflect natural conditions. Instead, experimental designs mimicking environmental fluctuations (Boyd et al. 2016) are needed to assess ecosystem sensitivity in a way that acknowledges the role of environmental heterogeneity for sensitivity of marine organisms to changing ocean properties. An ability to discriminate between holobiont stress response characteristics and forecast patterns of sensitivity response to OW will benefit

conservation decision-making by determining which marine habitats or species to protect as the climate changes.

1.3 Proteomics as a tool for global change research

The rapid development of 'omics' technologies have recently permitted better understanding of the molecular pathways underlying plastic phenotypic responses in corals and helped to identify candidate genes that may contribute to stress defense (Maor-Landaw and Levy 2016; Louis et al. 2017; Torda et al. 2017). In doing so, these studies demonstrated that data obtained from genome and proteome studies provides a basis for investigations targeting specific functions related to resilience and adaptability substantiating further research (Voolstra et al. 2015).

Variations in gene expression patterns (e.g., cDNA microarrays and mRNA sequencing) of a population in response to stress have been studied considerably (in corals but not LBF) to address the role of acclimatization through phenotypic plasticity (Maor-Landaw and Levy 2016). However, such transcriptomic studies rely on the risky and crude assumption that the corresponding proteins show equivalent trends (Mayfield et al. 2016b). To study functional processes and infer cellular behavior in organisms exposed to changing conditions, comprehensive high-throughput proteomics studies are more promising by giving insights into the entire molecular phenotype and its functional adaptations (Feder and Walser 2005). Using modern high-throughput proteomic techniques can highly improve experimental design, work-flow and quality of data, and has a high potential to play a prominent role in ecological and evolutionary studies (Diz et al. 2012). By performing such analysis parallel to direct measurements of physiological parameters, a better insight can be gained into why some species are more resilient to certain stressors and which adaptive processes may occur (Doo et al. 2014b; Tomanek 2014).

The 'PROTEOME' defines the entire set of PROTEins expressed by the genOME of an organism, tissue or cell at a specific time (Wilkins et al. 1996). It constitutes an equivalent concept to the 'genome', but is much more dynamic and can respond fast to changes in living conditions, stimuli or stressors (Tomanek 2014). As proteins are almost always the effectors of biological functions, studying their abundance (reflecting regulation and turnover) is a powerful tool to understand how the environment affects the biology of marine organisms (Tomanek 2011; Mayfield et al. 2016b). Likewise, proteome studies allow for detecting functional changes that can possibly improve the fitness of an organism (Silvestre et al. 2012).

1.3.1 Proteomics in photosymbiotic holobiont research

Aiming for the entire proteome, novel gel-free shotgun methods are less laborious and time-consuming, and allow for higher quantification power at relatively low costs compared to traditional approaches based on gel-electrophoresis (Oakley et al. 2016). Moreover, novel *in silico* technologies allow characterizing large amounts of proteins, even without having precise knowledge about the organism that produced them (Gotelli et al. 2012). The first coral and anemone studies utilizing mass spectrometry-based approaches were targeting only specific organic components (Peng et al. 2011; Drake et al. 2013; Ramos-Silva et al. 2013) or isolated the host proteins (Oakley et al. 2016; Ricaurte et al. 2016). Recent proteomic studies analyzed the entire coral tissue including symbionts (Weston et al. 2015; Garcia et al. 2016; Mayfield et al. 2016a, 2016b) and annotated the peptides/proteins *in silico* to either host or symbiont compartment. By applying homology-based search approaches the detected peptide sequences are matched to similar sequences of proteins in a database that contains host and symbiont genomes/proteomes, and thereby assigns it to the respective compartment of origin. This gives the rare opportunity to analyze and portray the response of a holobiont and simultaneously detect responses and interactions between its compartments (host and symbiont).

1.4 Research aims and approach

The overall goal of this thesis was to determine the effects of ocean warming on photosymbiotic foraminifera and to disentangle characteristics of the host and the symbionts that influence their adaptive capacities. To target the different mechanisms of stress responses, the following research questions were defined:

i) Are there differences in LBF thermal stress response between species or within one species living in different habitats, and how do these correspond to associated symbiont assemblages?

ii) Do temperature fluctuations in the form of single or episodic thermal peaks affect LBF the same way as chronic thermal stress, or do they induce acclimatization?

iii) What are the underlying cellular mechanisms of thermal stress responses in the host foraminifera and their symbionts?

1.4.1 Study organisms and their origins

The above questions were targeted by conducting a thermal stress experiment under controlled laboratory conditions on LBF of the family Amphistegenidae, belonging to the order Rotaliida in the supergroup Rhizaria. As one of the globally most abundant foraminifera in coral reefs the genus *Amphistegina* plays an essential role in these ecosystems (Hallock 1988, 2005; Hohenegger et al. 1999). Their lentil-shaped trochospiral tests are normally between 0.1 to 2 mm in diameter, and they usually show an olive-green coloration originating from their diatom symbionts (Figs. 1-1e, 1-2b and 1-5). After three to six months, they reach maturity and start either sexual reproduction by gamete broadcasting or asexual reproduction by multiple fissions, whereby hundreds of juveniles are released (Hallock et al. 1986). Due to this fast life cycle and relatively big size, *Amphistegina* species can locally reach very high densities of >1500 g m^{-2} and contribute more than 65% to the foraminiferal tests in reef carbonate sediments (Narayan and Westphal 2016). Their fate in future ocean conditions is consequently of great concern.

Although their ancestors are present in the geological record since 50 to 55 million years (Loeblich and Tappan 1988) and recent species exhibit high morphological plasticity (Hallock and Hansen 1978; Hallock et al. 1986; Toler and Hallock 1998), which both suggest high phenotypic plasticity and thus resilience against environmental changes, they were the first LBF observed to bleach in response to environmental stress (Hallock et al. 1993). Moreover, their common use as

bioindicators in past and present ecosystems is based on the assumption that the occurrence and physiological state of a species are directly linked to specific environmental conditions and thresholds. In order to improve their applicability as such we need to understand the factors that determine their occurrence, such as habitable temperature ranges.

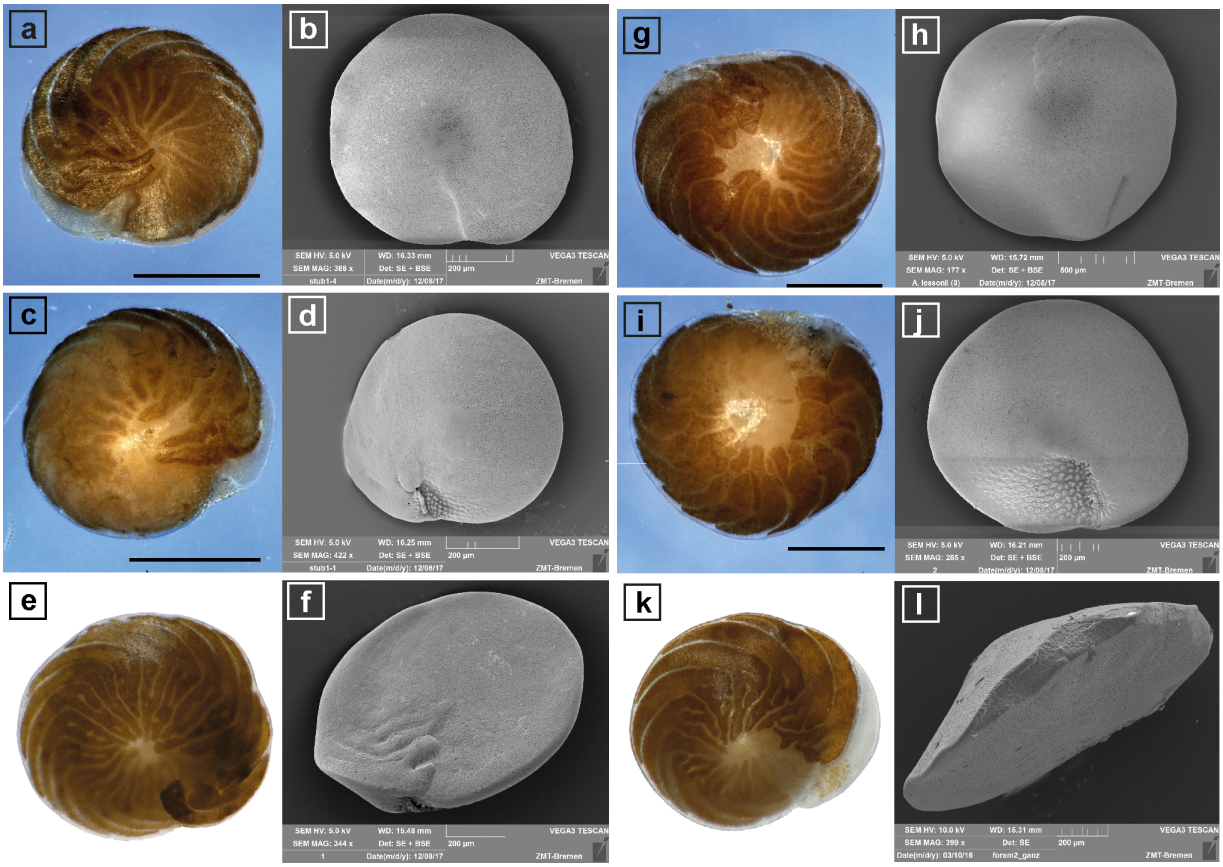

Figure 1-5. Light microscopy (scale bars: 0.5 mm) and SEM images of the studied foraminifera species: a, b, g, h, e–k) spiral side and c, d, I, j) umbilical side of (a–f) Amphistegina gibbosa *from the Florida Keys, USA, and (g–l)* A. lessonii *from Zanzibar, Tanzania; f) slightly inclined specimen showing the aperture; l) strongly inclined specimen that displays repair of a partly broken test.*

1.4.2 Experimental rationale and procedures

To address research question (i), three different populations (here referring to test groups that might, but do not necessarily need to represent populations in a biological sense) were included into the experiment and their responses to the different thermal stress treatments outlined below were compared (chapter 2). Two of them belonged to the species *Amphistegina gibbosa* (Fig. 1-5a–f) and were sampled from different depths on Tennessee Reef in the Florida Keys to serve for intra-species comparison. Both sampling sites are located closely together (~2.6 km) such that it can be assumed that both LBF populations belong to the same host species (genotypic), despite the lack of molecular identification tools for this genus. As they originated from different depths (18 m vs. 5 m below sea level; Figs. 1-6a–c and 1-6d–f, respectively), they have been exposed to discrete local habitat conditions during their previous lifetime. Consequently, due to these differences in thermal history both *A. gibbosa* populations may have acclimatized to dissimilar mean temperatures as well as amplitudes in temperature fluctuations. To screen for inter-species differences of the host, i.e., possible genotypic adaptations, *A. lessonii* (Fig. 1-5g–l) sampled from 5 m depth in a fringing reef near Zanzibar (Fig. 1-6g–i) were included as third population. Originating from the same depth, they were assumed to generally experience alike environmental conditions as the shallow-dwelling *A. gibbosa* population. Detected dissimilarities between stress responses could accordingly be linked to the foraminifer species. Lastly, a short fragment of the 18S rDNA was sequenced from individual foraminifera to identify the dominant symbiont and detect variations in symbiont assemblages between the three populations. If different symbionts possess dissimilar thermal preferences, the presence of different symbionts or a higher flexibility in symbiont association could assist holobiont acclimatization.

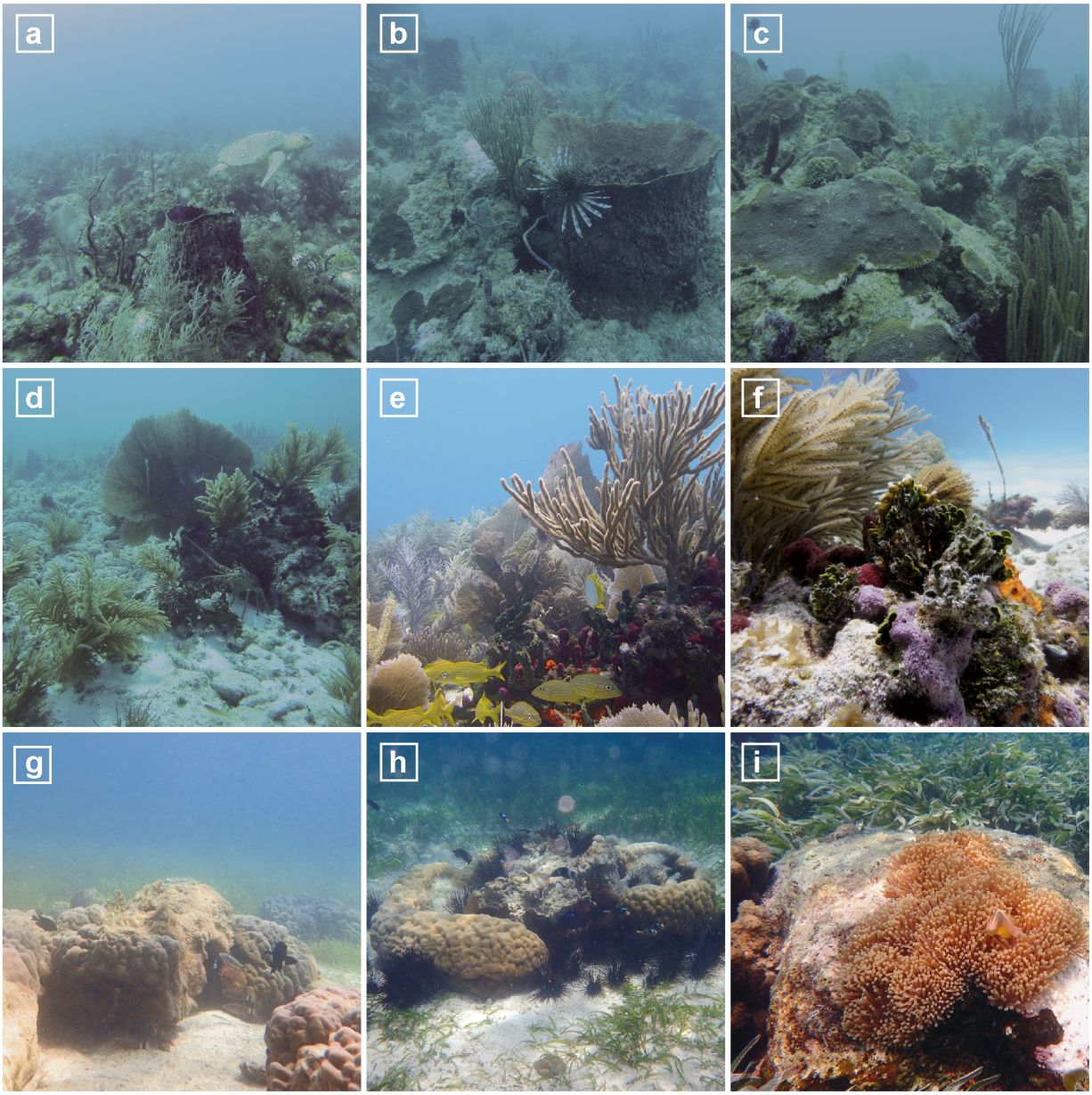

Figure 1-6. Photos of the three sampling sites: a) Tennessee Reef in the Florida Keys, USA, at 18 m depth in December 2014, and b–c) in June 2015, showing the common sponges and soft corals, as well as encrusting hard corals; d) Tennessee Reef in the Florida Keys at 5 m depth in December 2014, and e–f) in June 2015, showing high abundance of soft corals and gorgonians, sponges and calcifying algae, alternating with open sandy areas and occasional stony corals; g-i) the shallow patchy reefs close to Changuu near Zanzibar, Tanzania, at 5 m depth in April 2015 (credit: G. R. Narayan), showing mostly massive forms of hard corals, alternating with seagrass patches and open sandy areas.

The responses of hosts and symbionts were monitored during simulations of OW by temperature manipulations that included three different modes of thermal stress to address research question (ii). Over one month, all LBF were exposed to the following treatments that focused on different thermal stress scenarios exemplifying potential consequences of OW in coral reef habitats: (a) no stress, i.e., constantly 25.5°C, served as control; (b) a single three-day heating event up to 32°C, followed by control conditions, represented a transient thermal peak to induce thermal stress responses and observe whether host and symbiont physiologies would recover to pre-stressed conditions within three weeks; (c) four recurring three-day heating events up to 32°C, intermitted by six-day periods at control conditions, simulated episodic thermal peaks in order to monitor whether their impacts would change over time, i.e., if with every stress event the responses stay homogeneous then the intermitting thermal respite must facilitate recovery, if the response strength increases then the temperature alterations have an accumulative effect and do not allow for recovery, or if the response strength decreases then the fluctuating temperatures stimulate acclimatization to temporary thermal peaks; (d) chronic thermal stress that was increased to 32°C simultaneously to heating events, but remained constant until the end of the experiment, to observe the time resolved physiological adjustments to sustained OW in host and symbionts and thereby detect potential interactions or causations.

Variables that indicate either holobiont or symbiont performance were measured after every thermal stress event on the deeper-dwelling population of *A. gibbosa* as representative population to show how impacts of thermal stress develop over time (chapter 3). Nevertheless, distinguishing between the physiological responses of both symbiotic compartments is difficult in such tiny holobionts that cannot be physically separated, for example when measuring respiration rates. While several proxies (e.g., chlorophyll *a* concentrations, photosynthesis rates or RuBisCO expression) provide insights into symbiont performance, the host foraminifera are very difficult to be targeted in isolation. Hence, in order to understand how stress resilience in these holobionts and their single compartments function, tools and methods that allow distinguishing between host and symbiont physiology/phenology are necessary (Doo et al. 2014b), preferably on the basic molecular levels from genes to proteins. To answer research question (iii), the changes in the proteome of the representative population of *A. gibbosa* from 18 m depth in response to the different thermal treatments were analyzed (chapter 4). This allowed to complement the organism-level physiological parameters such as growth, motility, respiration and mortality by the underlying protein variations and hence elucidated the involved cellular processes.

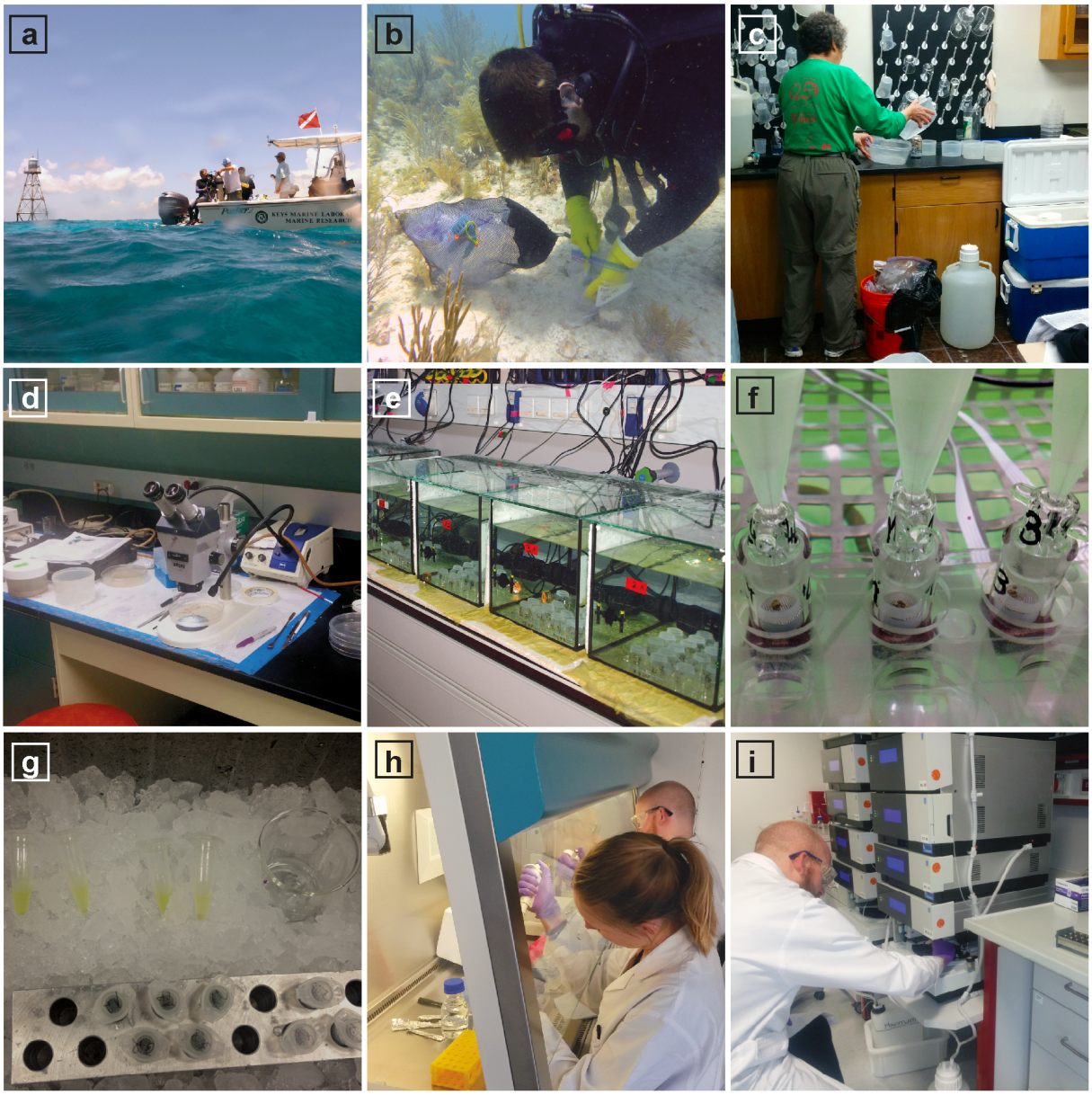

Figure 1-7. Some procedures undertaken for the presented thesis: a) sampling campaign close to the light house of Tennessee Reef by boat diving using SCUBA; b) coral rubble was collected and scrubbed into zip-lock bags; c) in the Keys Marine Laboratory, the contents of the plastic bags were transferred into screw-cap jars, whereby removing algae and organic debris; d) Amphistegina *spp. were picked from the resulting sediments under a microscope in the Bioindicators laboratory at the University of South Florida, St. Petersburg, USA; e) at the Marum laboratory in Bremen, the thermal stress experiments were conducted in 12 randomized aquaria, holding many glass vials that contained the different populations and subsets of foraminifera; f) oxygen consumption and production was measured with sensitive opdotes in small respirometry chambers submerged in a water bath. The foraminifera are placed on a mesh net that separates them from a tiny magnetic stirrer underneath; g) chlorophyll a was extracted from specimens using ethanol and measured photometrically; h) subsets of frozen specimens for proteome analysis were washed prior to protein extraction to minimize contaminations; i) tryptic digests of foraminifera and symbiont proteins are analyzed on a high precision liquid chromatography-tandem mass spectrometry system at the Leibniz Zentrum für Analytische Wissenschaften (ISAS), Dortmund, Germany.*

1.4.3 Applying proteomics to LBF

Enthused by the words of Tomanek (2014) 'Despite this high level of interest, there remain a number of obstacles to conduct proteomic analyses. […] The reward however, after navigating the challenges of a proteomics project, lies in the joy of becoming a molecular or biochemical ecologist, able to integrate multiple networks, e.g., tissue responses, and levels of biological organization', the analysis of the proteome of LBF holobionts was addressed for the first time to identify the roots of thermal stress response in both symbiotic compartments. The strategy was to use mass spectrometry-based high throughput proteomics technologies combined with an *in silico* separation of host and symbiont proteins to reveal compartment-specific responses of the holobiont to the different thermal stress scenarios. Such label-free approach estimates fold-changes in peptide abundances based on differences in ion currents among samples.

The attempt first required determination of the protein mass contained in an *A. gibbosa* specimen in order to estimate how many individuals are needed to reach the necessary biomass for a bottom-up proteomics workflow. Moreover, as no genomes from both compartments directly exist as reference databases, a database that contained all sequences publically available for foraminifera or closely related organisms within the Rhizaria and diatoms was assembled. While for diatoms, three genomes have been sequenced (Armbrust et al. 2004; Bowler et al. 2008; Lommer et al. 2012), only two foraminiferal genomes can be found. These belong to the giant naked freshwater species *Reticulomyxa filosa* (Burki et al. 2006; Glöckner et al. 2014) and the agglutinated Antarctic cold-water species *Astrammina rara* (Habura et al. 2011) that are rather distant relatives of the genus *Amphistegina*. Additionally, expressed sequence tags (ESTs) from various Rhizaria, mostly milliolids of the genus *Quinqueloculina,* are available (Burki et al. 2007). After adapting common shotgun proteomics workflows of sample extraction, cleaning and preparation to the small sample volumes enclosed by the thick calcareous test characteristic for Amphistegenidae, the abundances of all peptides common to all samples were measured.

To focus on those proteins that were reliably annotated and assigned to the right compartment with high certainty, iterative analysis and comparison of final outcomes were used to evaluate suitable cut-off thresholds for false discovery rates and amounts of unique peptides per protein. Afterwards, all proteins showing high similarity to each other were grouped into one protein cluster, further reducing the amount of proteins that needed to be assessed by statistical analysis, BLAST searches and annotation of molecular function and cellular processes. To decisively evaluate and verify the validity of this dual-compartment shotgun approach on a (or rather multiple) non-model organisms, the proteomics results were set into context and compared with the results of the physiological parameters measured on the

same population when addressing research question (ii). Ultimately, as the patterns of proteome responses induced by the different thermal stress treatments, which are indicated by significant abundance fold-changes of certain proteins with reference to the start of the experiment as well as to the control treatment, showed very high congruency to the physiological responses found in chapter 3, it was possible to draw conclusions on which functional changes and molecular processes are characterizing the host and symbiont cells in each of the applied OW scenarios.

1.4.4 Outline of the thesis

To target the defined research questions and achieve the overall goal to determine the effects of ocean warming on photosymbiotic foraminifera and disentangle characteristics of the host and the symbionts that influence their adaptive capacities, the individual chapters of this thesis focus on different aspects of thermal stress response (Fig. 1-8). Percentage contributions are given below the references and further details on personal contributions to each chapter can be found on the backside of the respective cover page.

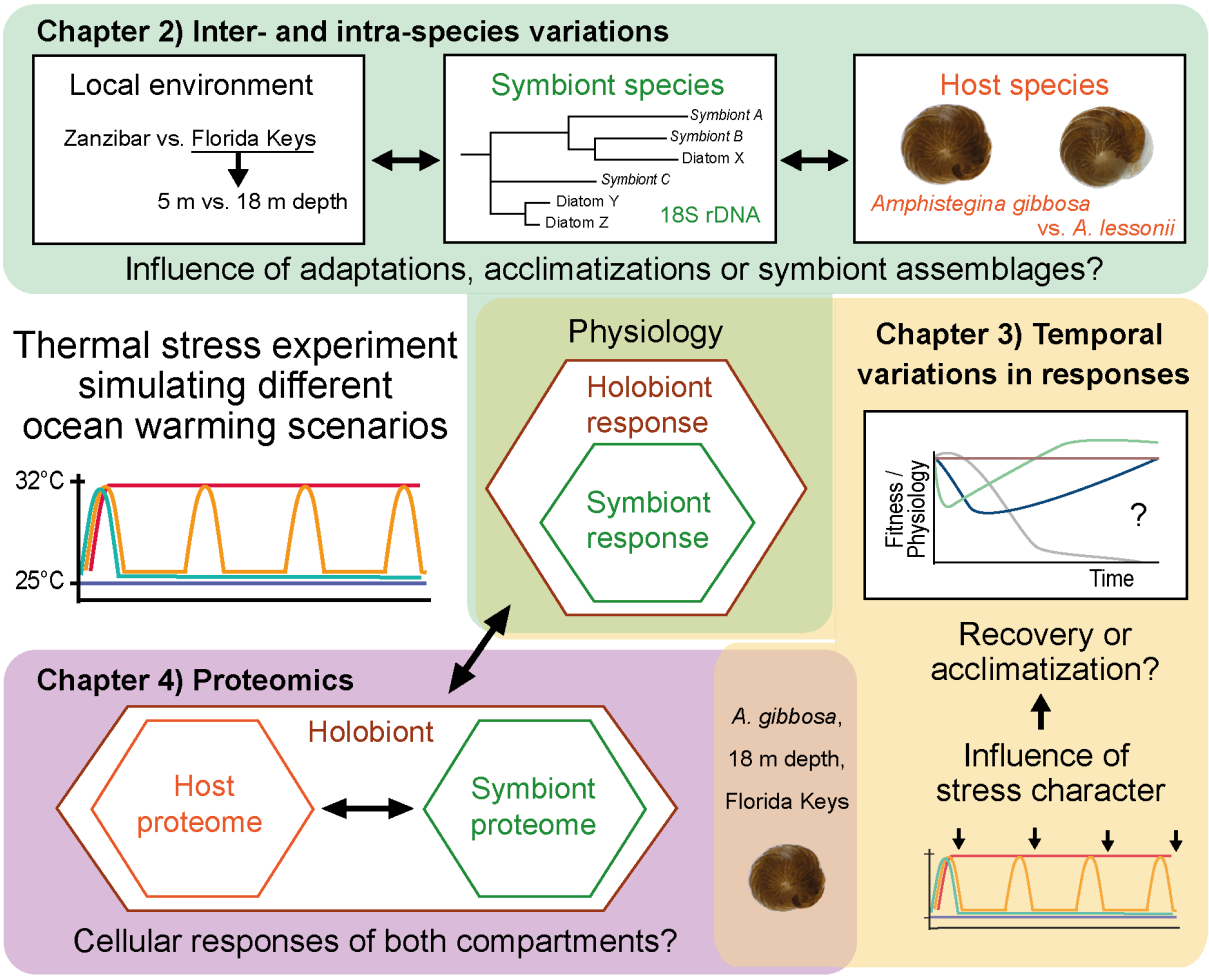

Figure 1-8. Concept of the presented cumulative thesis indicating holobiont and stress characteristics in the focus of the individual chapters, and how these were set into context with each other to study adaptive mechanisms that contribute to the resilience of photosymbiotic foraminifera.

Chapter 2: Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera *Amphistegina* linked to differences in symbiont type.
Stuhr M, Meyer A, Reymond CE, Narayan GR, Rieder V, Rahnenführer, J, Kucera M, Westphal H, Hallock P.
Contributions: Idea and concept: 90%; research: 70%; writing: 80%

Chapter 2 focuses on the inter- and intra-species variations in thermal stress responses. These are set into context to the associated symbiont assemblages and to the environmental conditions at the location of origin of each population. Two foraminifera species from different locations (*Amphistegina gibbosa*, Florida Keys, and *A. lessonii*, Zanzibar) serve to test for species-specific differences that may represent adaptations. Two *A. gibbosa* populations from different depth (5 m vs. 18 m) serve to test for intra-species variations that may indicate acclimatization to different environmental conditions. Their physiological responses to three different thermal stress scenarios and a control treatment are compared, including parameters indication holobiont and symbiont physiology. Potential influences of different adaptive traits in the associated symbiont assemblages are highlighted.

This manuscript is under review at *Coral Reefs*.

Chapter 3: Reef calcifiers are adapted to episodic heat stress but vulnerable to sustained warming.
Stuhr M, Reymond CE, Rieder V, Hallock P, Rahnenführer J, Westphal H, Kucera M.
Contributions: Idea and concept: 100%; research: 90%; writing: 90%

Chapter 3 focuses on the temporal variations in physiological responses to the different thermal treatments, which characterize distinctive stress scenarios. *A. gibbosa* from 18 m depth in the Florida Keys is used as representative population to discover changes over time in holobiont and photosymbiont performance. These may indicate recovery from a single pulsed stress event, existing adaptation or acclimatization to chronic and episodic thermal stress scenarios. In addition to the physiological parameters measured in chapter two, bleaching frequency and a biomarker assay determining total antioxidant capacity of the holobiont are included, giving further insights into the progression of effective cellular stress defense against reactive oxygen species.

This manuscript has been published in *PLoS ONE*.

Chapter 4: Disentangling thermal stress responses in reef-calcifiers and their photosymbionts by shotgun proteomics.
Stuhr M, Blank-Landeshammer B, Reymond CE, Kollipara L, Sickmann A, Kucera M, Westphal H.
Contributions: Idea and concept: 90%; research: 80%; writing: 90%

Chapter 4 focuses on the underlying cellular responses of the host and the photosymbiont compartment in the representative population of *A. gibbosa* probed in chapter three. A bottom-up proteomics approach adapted for photosymbiotic foraminifera allows for the first time disentangling the contributions of both compartments. The determined changes in protein abundances indicate regulation of various cellular processes involved in different metabolic pathways and proteome stress responses, which are set into context with the physiological holobiont and photosymbiont stress responses discussed previously. The influence of the character of the stress is further defined by the resulting patterns of protein variations. The interpretation of protein regulations and their attributed molecular functions result in a model that suggests the underlying cellular mechanisms of temperature-induced bleaching in photosymbiotic foraminifera.

This manuscript is under review at *Scientific Reports*.

These chapters are followed by the extended discussion (Chapter 5) that connects the aforementioned studies and sets them into the context as outlined in Fig. 1-8, discussing additional aspects and summoning up the main results of this thesis. A subsequent outlook (Chapter 6) highlights emerging questions and potential future research lines.

To enhance clarity, the list of all references for the individual chapters is provided at the end of this thesis.

Chapter Two

VARIABLE THERMAL STRESS TOLERANCE OF THE REEF-ASSOCIATED SYMBIONT-BEARING FORAMINIFERA *AMPHISTEGINA* LINKED TO DIFFERENCES IN SYMBIONT TYPE

This work is under review at *Coral Reefs*

Stuhr M[1], Meyer A[1], Reymond CE[1], Narayan GR[1], Rieder V[2], Rahnenführer J[2], Kucera M[3], Westphal H[1,4], Hallock P[5].

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Personal contribution to chapter 2:

Idea and concept:
M. Stuhr developed the idea of combined inter- and intra-species comparison of thermal stress responses to detect differences in adaptation or acclimatization, with contributions from C. Reymond, H. Wesphal and M. Kucera.
M. Stuhr sampled the foraminifera from Florida with the aid of P. Hallock, and G. R. Narayan sampled the foraminifera from Zanzibar.

Research:
M. Stuhr carried out the experiment and conducted physiological sample analysis.
A. Meyer extracted symbiont sequences, analyzed and plotted the results with contributions from M. Stuhr.
V. Rieder, J. Rahnenführer and M. Stuhr analyzed the data and created plots and tables.
M. Stuhr interpreted the data.

Writing and figure preparation:
M. Stuhr wrote the initial manuscript and prepared the figures, with contributions on method descriptions from A. Meyer and V. Rieder.
P. Hallock, H. Westphal, V. Rieder, C.E. Reymond, and M. Kucera made improvements.

Abstract

Adaptation, acclimatization, and symbiont diversity are known to regulate thermal tolerance in corals, but the role of these mechanisms remains poorly constrained in other photosymbioses, such as large benthic foraminifera (LBF), which are known to bleach at temperatures that are likely to be exceeded in the near future. LBF inhabit a broad range of shallow-water settings. Within species, differences in thermal tolerance have been found among populations from different habitats, but it is not clear whether such differences occur among LBF inhabiting similar habitats, but differing in other aspects, such as symbiont type. To this end, we compared responses to thermal stress in specimens from a population of Amphistegina lessonii, *an abundant Indo-Pacific species, to specimens of* A. gibbosa, *its Atlantic counterpart, from a similar environment but two different water depths (5 m and 18 m). Test groups of each species were exposed in a common experiment to three thermal stress scenarios over a four-week period. Growth, respiration, mortality, and motility were measured to characterize the holobiont response. Coloration, photosynthesis, and chlorophyll a content were measured to determine the response of the endosymbiotic diatoms. The photosymbionts were characterized by genetic fingerprinting. Our results show that, although groups* of A. gibbosa *were collected from different habitats, their responses were similar, indicating only marginally higher tolerance to thermal peaks in specimens from the shallower site. In contrast, species-specific differences were stronger, with* A. lessonii *showing higher tolerance to episodic stress and less pronounced impacts of chronic stress on motility, growth and photosymbiont performance. These inter-species variations are consistent with the presence of different and more diverse symbiont assemblages in* A. lessonii *compared to* A. gibbosa. *This study demonstrates the importance of considering symbiont diversity in the assessment of intra- and interspecific variations in stress responses in LBF.*

2.1 Introduction

Ocean warming, manifested by increase in mean temperature and higher frequency of extreme heat events (IPCC 2013), has severe impacts on coral-reef ecosystems (Hoegh-Guldberg et al. 2017). Identifying factors that contribute to resilience of photosymbiotic reef organisms to is essential to evaluate their response to further warming (Barshis et al. 2010; Howells et al. 2011). Thermal exposure history may lead to local acclimatization or ultimately induce adaptation (Middlebrook et al. 2008; Oliver and Palumbi 2011b). In photosymbiotic taxa, resilience may be promoted by switching to more heat-resistant symbionts or harboring an assemblage of symbionts with different thermal tolerance (Baker 2003; Oliver and Palumbi 2011a). These mechanisms have been primarily studied in corals, but they appear to also apply for other photosymbiont-bearing calcifiers such as foraminifera (Momigliano and Uthicke 2013; Prazeres et al. 2016b, 2017a; Schmidt et al. 2016b).

Facilitated by symbioses with a range of microalgae such as dinoflagellates and diatoms, large benthic foraminifera (LBF) can locally reach extremely high population densities and contribute approximately 5% to reef-carbonate production (Langer 2008). They are critical for the formation, maintenance and stabilization of reef habitats, beaches, low-lying coral-sand cays or reef islands (Hohenegger et al. 1999; Yamano et al. 2000; Doo et al. 2012a). Moreover, they provide useful bioindicators for the assessment of water quality in reef environments (Hallock et al. 2003; Cooper et al. 2009). Their long evolutionary history makes them important paleoecological proxies, but also highlights the question of how they were able to persist and thrive through phases of strong environmental variations (Hallock and Pomar 2009). With respect to the current climate change, their adaptive potential to ocean warming is of particular interest.

The foraminiferal host is dependent upon its symbiotic algae for growth and calcification (Hallock 2000). As in corals, this photosymbiotic relationship may be disrupted by stressful conditions such as high light intensities or temperatures, leading to bleaching (Hallock et al. 2006; Schmidt et al. 2011). Thermal tolerances clearly vary among LBF belonging to different lineages (Schmidt et al. 2011, 2016b; Engel et al. 2015), and thermal tolerances also appear to differ among populations of the same species from different habitats (Prazeres et al. 2016b, 2017b). However, the use of different experimental setups makes it difficult to compare the role of local habitat acclimatization with differences due to other factors, such symbiont type. To date, no experiments have been carried out to simultaneously test intra- and inter-species variations of LBF in response to different thermal stress scenarios, while also assessing the associated photosymbiont assemblage.

For an inter-species comparison of two closely related taxa, the widely distributed Indo-Pacific species *A. lessonii* was selected for comparison with its western Atlantic-Caribbean counterpart *A. gibbosa*. Similar habitat preferences, depth distributions and test morphologies of *A. gibbosa* and *A. lessonii* indicate that they fill comparable ecological niches (Hallock et al. 1986; Langer and Hottinger 2000). Moreover, Hallock (1999) considered them sibling species and Barnes (2016) postulated that *A. gibbosa* arose from *A. lessonii* ancestors that were isolated on the Caribbean side after the closing of the Panama seaway.

Amphistegina harbor endosymbiotic diatoms (Lee 2006) and are found globally in association with reefs and carbonate shelves (Hallock 1999; Hohenegger et al. 1999). *Amphistegina* have been found in the geologic record for more than 50 million years (Loeblich and Tappan 1988), and have since significantly contributed to the production of tropical carbonate shelf sediments. Moberly and Chamberlain (1964) reported that foraminiferal shells accounted for approximately 27% of the beach sands on the island of O'ahu, Hawai'i, USA, of which Muller (1976) estimated

that approximately 90% was produced by *Amphistegina* spp.. In the shallow-water areas around Zanzibar, Tanzania, *A. lessoni* and *A. lobifera* contribute up to 70% of the foraminiferal shells in reef-wide sediments (Shaghude et al. 2002; Narayan and Westphal 2016).

In addition to the interspecific comparison, to test if responses within one species are related to local acclimatization, specimens of *A. gibbosa* were collected from two depths at the same locality. In a one-month experiment, the three test groups were exposed to (i) no thermal stress, i.e., control conditions at constant 25.5°C; (b) a single thermal stress event up to 32°C for three days, followed by control conditions; (c) episodic thermal stress events alternating with periods of six days at control conditions, and (d) chronic thermal stress at 32°C over one month, in an experimental setup described by Stuhr et al. (2017) (Fig. S1). Variations in photosymbiont and host performance in response to the different treatments were determined by monitoring physiological variables including chlorophyll *a* concentrations (Chl *a*), *L*a*b** color values, oxygen production and consumption rates, growth, motility and mortality. To assess the influence of symbiont type, the diatom symbionts of each test group were identified genetically

This experimental setup addresses the question whether species-specific predetermined ecological ranges dominate over intra-species acclimatization to local habitat, and if variations in thermal stress responses relate to specific symbiont assemblages. This has implications for the application of LBF as (past and present) reef indicators, because if tolerances vary, thresholds found in experimental studies cannot be extrapolated to other populations. Likewise, the assessment of environmental conditions as well as projections on the fate of LBF and other photosymbiotic calcifiers in future oceans would have to take local or species-specific acclimatization capacities into account.

2.2 Materials and methods

2.2.1 Field collection

To allow intra-species comparison of thermal tolerance, *A. gibbosa* were sampled in June 2015 on Tennessee Reef (Fig. 1a) in the Florida Keys, North Atlantic, from 5 m depth (24°44'41.67"N, 80°46'55.82"W) and from 18 m depth (24°45'8.33"N, 80°45'26.33"W). Environmental parameters of the locations were described previously (Hallock et al. 1995; Mendez-Ferrer et al. 2018). Mean water temperatures in the Florida Keys are around 26.5°C, ranging from winter minima of 21°C to summer maxima of ~30.5°C. At depth, the magnitude of diurnal and faster thermal fluctuations typical for the area is higher than close to the surface, but temperatures are generally lower and peak values >29°C occur rarely (Leichter et al. 2006). Other differences between the sites include irradiance, wave exposure and

influence of waters from Florida Bay (e.g., Baker et al. 2009; Mendez-Ferrer et al. 2018).

Amphistegina lessonii was sampled in April 2015 off Zanzibar (Tanzania) in the western Indian Ocean near the small island of Changuu in a shallow patch reef and seagrass meadow at approximately 5 m depth (6°07'13.66"S, 39°09'43.08"E) (Fig. 1b). Water temperatures in 3 m depth around Zanzibar are typically 26–28°C for most of the year, and usually reach their maximum around 29°C in March (Muhando 2002). Mixed semidiurnal tides with mean spring amplitude of 3.3 m influence the shallow habitats that were formerly described (Bergman and Öhman 2001; Narayan and Westphal 2016).

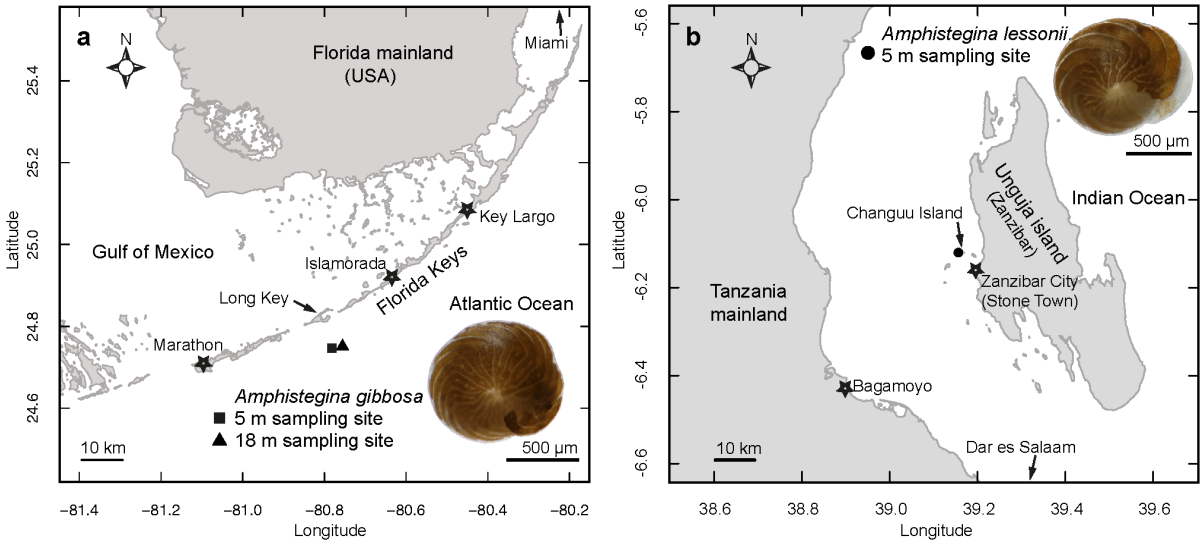

Figure 2-1. Maps indicating collection sites and including photos of (a) Amphistegina gibbosa *from 5 m (square) and 18 m (triangle) depth, in the Florida Keys, USA, and (b)* A. lessonii *(circle), near Changuu Island off the coast of Zanzibar, Tanzania.*

Sample collection and culturing were performed as described in Stuhr et al. (2017). Briefly, the specimens were collected from pieces of coral rubble, brought to the field-based laboratories and separated from the sediments (Hallock et al. 1986). They were stored under stock-culture conditions, i.e., ~25°C at low light, until transportation within less than 24 h in insulated containers to Bremen, Germany. Prior to the experiment, all specimens were acclimatized to laboratory conditions of 25.5 ± 0.5°C and 5–12 µmol photons m^{-2} s^{-1} for at least three weeks. All test groups were kept in aquaria filled with synthetic seawater (Tropic Marin Sea Salt, Germany) at salinity 35.5, and fed by adding 15 µl of autoclaved microalgae every nine days (Schmidt et al. 2016a).

2.2.2 Experimental design and analysis

The experimental settings and analytical methods were described in detail previously (Stuhr et al. 2017). The experiment was carried out in 12 independent aquaria. Three randomly allocated replicate tanks were used for each of the four different thermal treatments: the 'Control' remained at constant 25.5 ± 0.5°C. For the 'Single stress' event a temperature peak up to 32°C over three days was simulated. 'Episodic stress' was induced by repeating the temperature peak every nine days, with six days at control conditions in between. 'Chronic stress' was exerted by constant temperature of 32 ± 0.5°C. After 30 days, thus after the fourth stress event in the episodic-stress treatment, the experiment was terminated (see Fig. 2 in Stuhr et al. 2017).

The same subset of five specimens per treatment and group in each aquarium was examined before the experiment and at the end of the experiment to determine motility, mortality, coloration, growth, respiration and photosynthesis. Specimens that appeared dead were excluded from further analysis, but remained within the experiment. For Chl *a* analyses, sub-samples were taken from the aquaria and frozen at –80°C until further processing. To provide supplementary insights into physiological adjustments over time, color, growth, mortality and motility were also recorded and subsamples for Chl *a* analysis in *A. gibbosa* were taken after each temperature-stress event, i.e., on days 4, 13 and 22. Motility was used as an indicator for the foraminifers' activity (Schmidt et al. 2011) and estimated by movement (dislocation) since the last sampling event (Stuhr et al. 2017). The proportion of empty shells was recorded during sampling, representing mortality, which can result from reproduction, stress-induced death or unknown causes.

To estimate growth rates and document changes in coloration, high-resolution photographs were taken with fixed settings on a standardized background. Images were analyzed to calculate daily growth rates, based on increases in surface area (spiral side) of all surviving foraminifera (ter Kuile and Erez 1984) and to determine holobiont color in the CIE *L*a*b** color space (Hosono et al. 2012). The resulting CIE color space values represent: *L** = whiteness (brightness), *a** = position between green (–) and magenta (+), and *b** = position between blue (–) and yellow (+). Respiration (oxygen consumption) and photosynthesis (oxygen production) were determined by measuring changes in oxygen concentrations in ~1 ml respiratory chambers, and gross photosynthesis was calculated from their difference. After a dark-acclimation period, respiration was measured for 30 min during dark incubation, followed by a 30-min light phase for net photosynthesis measurements (Schmidt et al. 2011; Fujita et al. 2014; Stuhr et al. 2017). Chlorophyll *a* concentration was measured on subsamples containing ~3 specimens. After extraction in ethanol, absorbance was measured at 665 nm and 750 nm using a

plate reader. The resulting Chl *a* contents were normalized by the weights of the dried foraminiferal pellet (Schmidt et al. 2011; Stuhr et al. 2017).

2.2.3 Data analysis of physiological response parameters

To illustrate the differences between treatments and test populations at the end of the experiment, bar plots with corresponding error bars were drawn, representing the mean values with double standard error (SE). Except for mortality data, variable F-tests were used for the pairwise comparison of single treatments, with a global level of significance <0.05. In addition to all pairwise comparisons, more general hypotheses recognizing changes in mean value between more than two independent samples are required. The latter are called intersection hypotheses. In a closed testing procedure (Marcus et al. 1976), all elementary and intersection hypotheses are tested with a local significance value. Each elementary hypothesis can be rejected at the global-significance value if, in addition to the elementary hypothesis, all intersectional hypotheses including the elementary hypothesis are rejected at the local level of significance. A closed testing procedure is beneficial since there is no need to adjust the global level of significance for single hypotheses. As described previously (Stuhr et al. 2017), repeated-measures analysis of variance (ANOVA) was carried out on the variables where time-resolved data were available.

Logistic regression, instead of linear modeling and two-sided F-tests, was used for the binary outcome 'mortality' (dead or alive). By means of a likelihood-ratio test significant changes among treatments were tested. The closed testing procedure was used analogously, using R (R Core Team 2016).

For multivariate analysis of all physiological variables measured at the end of the experiment, proportional mortality data were arcsine transformed. A principal component analysis (PCA) based on a correlation matrix and a discriminant analysis were conducted using PAST3 (Hammer et al. 2001). Missing values were treated by iterative imputation in the PCA and column-average substitution in the discriminant analysis. Row-wise bootstrapping was carried out (n = 99) to construct 95% confidence intervals.

2.2.4 Molecular analysis of photosymbionts

Subsets of specimens were collected from the acclimation aquaria one week before the start of the experiment and transferred into a Tris-HCl (100 mM) buffer containing EDTA (2 mM) and $MgCl_2$ (5 mM). The subsets included four from *A. gibbosa* (Ag t0) and two from *A. lessonii* (Al t0), with several individuals pooled in each extraction. To identify their symbionts at the end of the experiment, 19 specimens were sampled with at least one individual from each treatment and test groups. Single specimens (six *A. gibbosa* from 18 m, seven *A. gibbosa* from 5 m,

and six *A. lessonii* from 5 m) were placed into vials without water or buffer. All samples were frozen and stored at –80°C until DNA extraction, which used a CTAP (AppliChem) / Proteinase K (20 mg/µl) digestion step with subsequent phenol chloroform extraction (Green and Sambrook 2012). All samples were pulverized using a micropestle in 500 µl lysis buffer with 5 µl proteinase K and incubated for 2 h at 60°C in a thermo shaker. After digestion, shell and cell debris were centrifuged, 450 µl supernatant were transferred into phenol:chloroform (1:1) and well mixed by shaking, followed by a 10 min centrifugation step. Then, 300 µl of the upper phase were mixed with 250 µl isopropanol plus 0.5 µl vivid violet (Roboklon), incubated for 30 min at room temperature and stored at –20°C overnight. After 10 min centrifugation, the pellet was washed with 70% ethanol. All centrifugation steps used 10,000 *g*.

Amplification of a 442 bp fragment of the 3' end of the 18S rDNA of the LBFs photosymbionts was done using the primers SymFS1 and 1528R (Schmidt et al. 2015) and either Taq (Roboklon) or Phusion (Thermo Scientific) polymerase with the following cycling conditions: 95°C for 2 min, 35 × (98°C 10'', 56°C 30'',72°C 1 min) and final elongation at 72°C for 5 min. PCR products were SAP digested and Sanger sequenced (starseq, Mainz) using the SymF1 primer. Three PCR products from Ag18, one Ag5 and one Ag t0 were heterogeneous and had to be cloned for sequencing using the TOPO-TA cloning kit (Thermo Scientific) with one shot chemical competent TOP10F' cells following manufacturer's recommendations. Up to 5 clones were Sanger sequenced using the M13F primer (starseq, Mainz) until at least one diatom sequence was recovered. The resulting diatom sequences were deposited at ENA under the accession numbers LT976804 to LT976830. The sequences suspected as contaminants are listed in Table S7.

Sequences were trimmed for primers, doubtful bases and vector contamination using Geneious 8.1.9. Alignments were done using T-coffee (Notredame et al. 2000) after adding sequences with close BLAST hits or from diatoms typically found in LBF (Lee 2006; Barnes 2016; Schmidt et al. 2016a; Prazeres et al. 2017a). Alter (Glez-Peña et al. 2010) was used to condense haplotypes with identical sequences. The taxonomic assignment was done by calculating a Maximum Likelihood phylogenetic tree in MEGA6 (Tamura et al. 2013) using the default parameters except applying the Gamma distribution instead of uniform substitution rates.

2.3 Results

Results indicating photosymbiont performance are displayed in Fig. 2, while holobiont variables are shown in Fig. 3. All F-test outcomes are summarized in the Table S1. Additional figures showing results for both groups from 5 m depth (Ag5 and Al5) at several time steps (after each episodic stress event) and the results of repeated-measures ANOVA complemented by Tukey's HSD post hoc test are given

in the supplementary material (Figs. S1 and S2, Tables S2-S6). Equivalent data for *A. gibbosa* from 18 m depth (Ag18) can be found in Stuhr et al. (2017). Chronic thermal stress had the strongest effect on all test populations (Figs. 2–4). Episodic and single-stress events induced minor responses in different parameters, which varied among the three populations. Most variables also changed in the control treatment over the duration of the experiment. This change was of a comparable magnitude to the differences between single- and multiple stress treatments and is likely the result of the continued ontogenetic development of the foraminifera over the one-month duration of the experiment (Stuhr et al. 2017). Since this influence affected all test populations equally, it did not obstruct the outcome of the study and was not further examined.

Table 2-1. P-values of two-sided F-Tests for the different populations of Amphistegina *species exposed to different thermal stress treatments: A closed testing procedure is used and the values shown here refer to the intersection hypotheses comparing all four treatments. Results of additional hypothesis are shown in Table S2-1. P-values are marked* ***bold*** *if corresponding hypotheses are rejected at the* ***global*** *level of significance.*

Variable	*A. gibbosa*, 18 m	*A. gibbosa*, 5 m	*A. lessonii*, 5 m
*L** value	0.082	0.098	**0.012**
*a** value	**0.017**	**0.020**	**0.008**
*b** value	**< 0.001**	**0.004**	**0.034**
Chlorophyll *a* concentration	**< 0.001**	**< 0.001**	0.223
Net Photosynthesis	**0.001**[1]	0.080	**0.045**
Gross Photosynthesis	**0.001**[1]	0.837	**0.007**
Respiration	0.122[1]	**0.035**	**0.016**
Growth	0.066	**0.002**	**0.012**
Motility	0.367	0.340	0.703
Mortality	**0.001**	**0.006**	1.000

[1] Episodic-stress treatment not included, because values are missing.

2.3.1 Symbiont performance

The overall responses of the symbionts to the different thermal-stress scenarios were similar among the test populations, with the most distinct responses to chronic thermal stress, but the extent of symbiont damage differed between species. Symbiont performance of *A. gibbosa* sampled from 18 m depth strongly resembled those of the same species sampled at 5 m depth, while the magnitude of change as well as the variables that were significantly affected were different in *A. lessonii* (Tables 1 and S1).

Amphistegina gibbosa exposed to chronic stress changed from their natural golden-olive to a light greenish-yellow coloration, as indicated by *a** and *b** values (Fig. 2b–c). Likewise, Chl *a* concentrations decreased (Fig. 2d), though *L** values were not significantly affected (Fig. 2a). Net photosynthesis rates varied strongly (Fig. 2e) and showed negative values under chronic stress, however, differences

were only significant on the local level. Gross photosynthesis responses to the different treatments also varied (Fig. 2f). Among all variables indicating symbiont performance, photosynthesis appeared to differ most between the two groups of *A. gibbosa*. However, this might be due to the missing values for episodic stress in Ag18 and this difference should not be over-interpreted. Over the course of the experiment, colors across treatment in both *A. gibbosa* populations changed in similar ways. Chl *a* concentrations of Ag18 in the single-stress treatments were similar to those under episodic stress, while in Ag5 they behaved similarly to control-treatment concentrations. In contrast to Ag18, *L** and *a** values of Ag5 showed no significant interactions between time and treatment, but all other ANOVA results indicate similar response patterns.

Amphistegina lessonii from Zanzibar showed stronger changes in coloration and photosynthesis, although Chl *a* concentrations were not significantly different among treatments (Fig. 2d). *L*a*b** color values were affected at the global level in the intersection comparison of all treatments (Table 1), but no differences were detected in the direct pairwise comparison of single treatments (Fig. 2c). In the other treatments, color values remained similar over time (Fig. S2a–c). Corresponding to the overall higher Chl *a* concentrations, *A. lessonii* was generally darker and greener, i.e., had lower *L** and *b** but higher *a** values than *A. gibbosa*. Because net photosynthesis was highest in the stress-event treatments (Fig. 2e), the chronic-stress treatment was significantly reduced compared to the single-stress (global) and the episodic-stress treatment (local), but not to the control. Gross photosynthesis rates indicate a significant reduction of symbiont performance under chronic stress (Fig. 2f).

2.3.2 Holobiont performance

Thermal stress responses of the holobionts differed between species and between the two populations of *A. gibbosa*. Respiration rates differed significantly between treatments in both test populations from 5 m depth (Table 1), but the directions of change were opposite between species (Fig. 3a). The *A. gibbosa* groups showed significant increases under chronic thermal stress at the global level and also after a single-stress event at the local level (Table S1). In contrast, *A. lessonii* was negatively influenced by both treatments.

Mortality, which could represent death or reproduction, showed significant species- and treatment-specific responses. Mortality was markedly prevalent in *A. gibbosa* in both the control and the single-stress treatments, but remained rare in episodic- and chronic-stress treatments in *A. gibbosa*, and was absent in *A. lessonii* in all treatments (Fig. 3b, Table S2). Also, growth differed significantly in both test groups from 5 m depth (Fig. 3c), with a severe reduction under chronic stress in both species. While *A. gibbosa* grew continuously less over time (Fig. S1 & Stuhr et

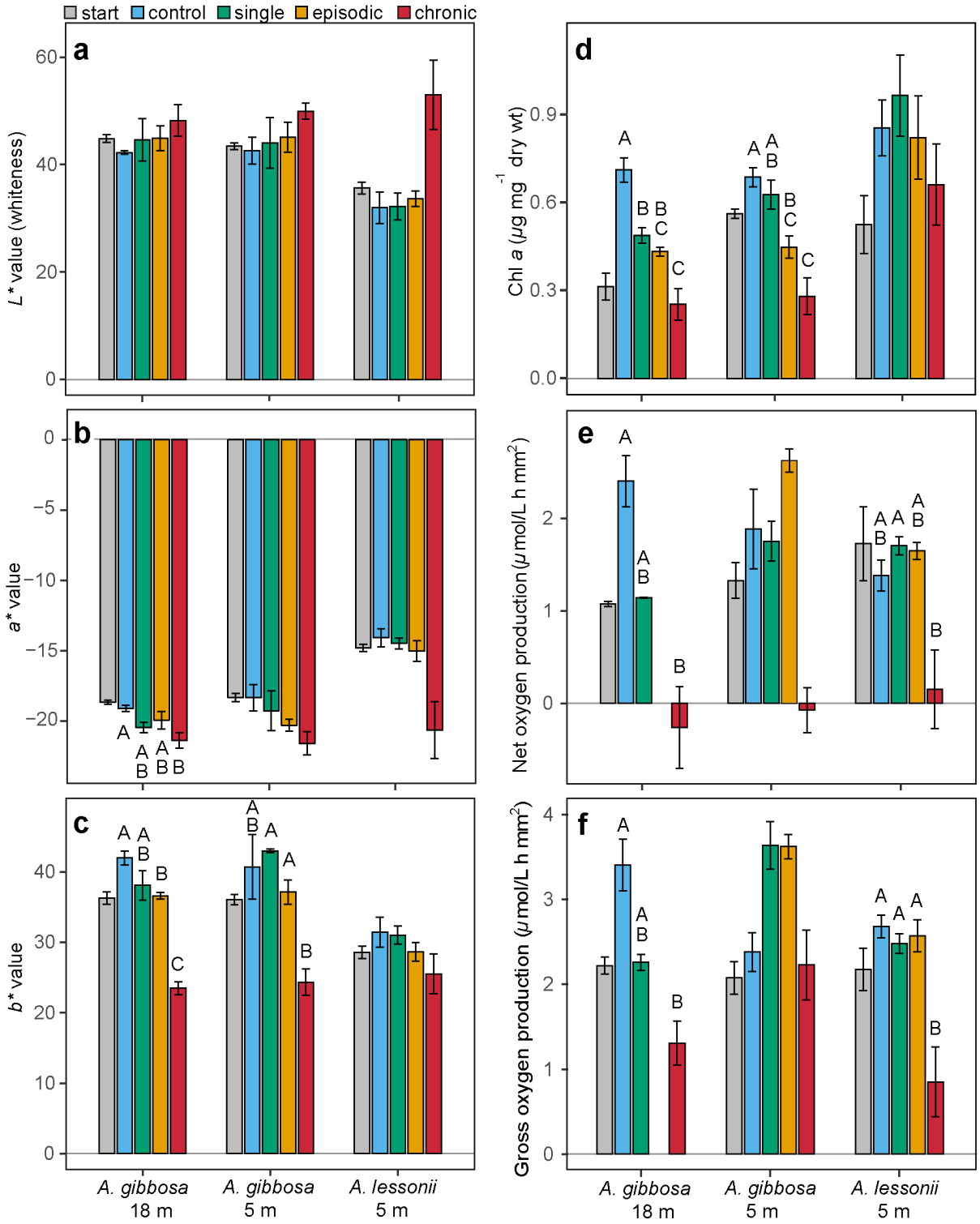

Figure 2-2. Means and SE (n = 3) of (a-c) CIE L*a*b* *color space values, (d) chlorophyll* a *concentrations, (e) net photosynthesis, and (f) gross photosynthesis (values for the episodic-stress treatment of* A. gibbosa*, 18 m, are missing due to computer failure) of* Amphistegina gibbosa *from 5 m and 18 m depth and* A. lessonii *from 5 m depth at the start of the experiment (grey) and after exposure to no stress/control (blue), a single stress event (green), episodic stress events (yellow) or chronic thermal stress (red). Color values represent:* L* *= whiteness,* a* *= green (-) to magenta (+),* b* *= blue (-) to yellow (+). Values from the start of the experiment are included in the bar plots for completeness, but were not included in the statistical analysis. Different capital letters indicate significant differences in the pairwise comparison between two treatments on the global level.*

al. 2017 Fig 4c), growth rates of *A. lessonii* dropped within the first three days of the experiment and remained stable after the initial decrease (Fig. S2d). Motility was not affected globally in any population (Fig. 3d), but reductions at the local level were detected for both *A. gibbosa* populations under chronic stress. Consequently, treatment and the interaction of treatment and sampling day significantly influenced motility of *A. gibbosa*.

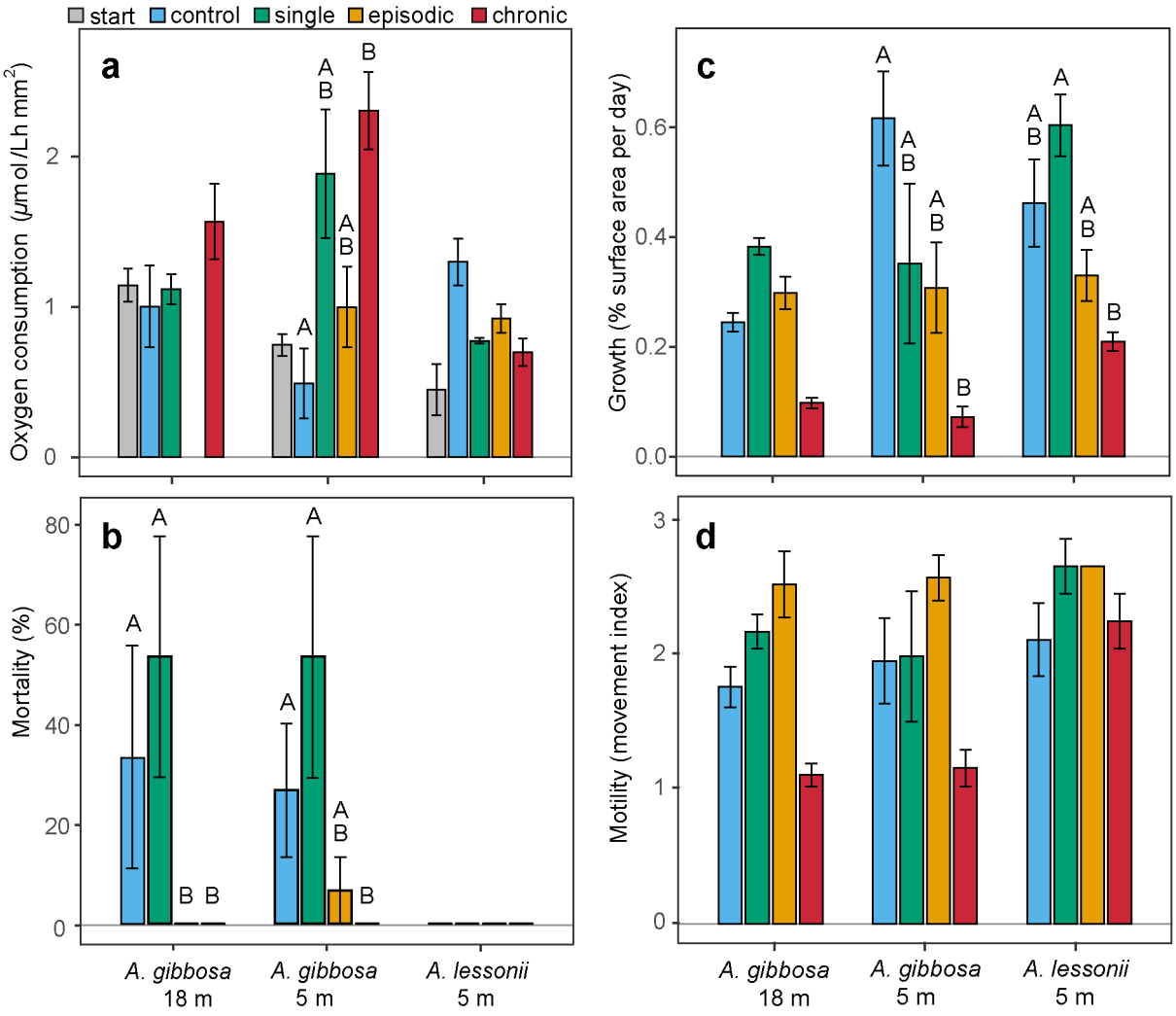

Figure 2-3. Means and SE (n = 3) of (a) respiration rates, (b) mortality, (c) growth and (d) motility of Amphistegina gibbosa *and* A. lessonii *from 5 m and 18 m depth after exposure to no stress/control (blue), a single stress event (green), episodic stress events (yellow) or chronic thermal stress (red). Respiration rates from the start of the experiment (grey) are included in the bar plots for completeness, but were not included in the statistical analysis (values for the episodic-stress treatment of* A. gibbosa*, 18 m, are missing due to computer failure). Different capital letters indicate significant differences in the pairwise comparison between two treatments on the global level.*

Multivariate analysis of all variables measured at the end of the experiment confirmed a consistent reaction among replicates and revealed patterns of separation among treatments and between species (Fig. 4). Higher Chl *a*, absence of mortality and differences in color space values *L** and *a** distinguished *A. lessonii* replicates from *A. gibbosa* test groups and replicates. In contrast, the chronic-stress

treatments produced a distinct cluster, indicating similar response of nearly all replicates in all populations. Higher respiration rates, *b** value changes and growth reductions were parameters that characterized specimens subjected to chronic-stress treatments.

In a multigroup discriminant analysis of all treatments for the three populations, the two species could be completely separated along the first axis (Fig. S3), which accounted for 94% of the variability and was predominantly influenced by differences in Chl *a* and *L*a*b** values. The test populations of *A. gibbosa* were minimally separated along the second axis, which accounts for only 6% of the variability.

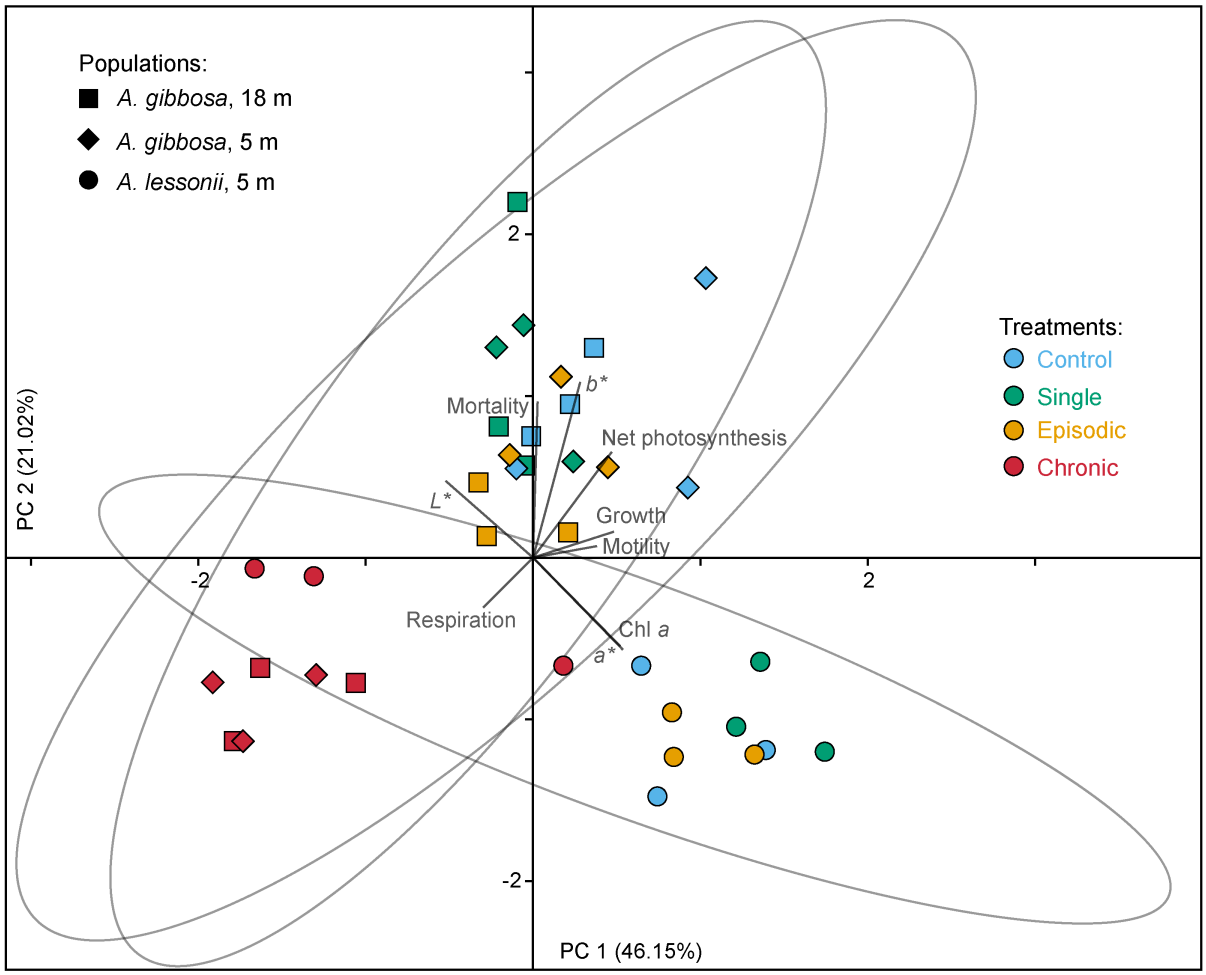

Figure 2-4. Principal component analysis including 95% ellipses for each population of Amphistegina gibbosa *from the Florida Keys at 18 m (squares) and 5 m depth (diamonds), and* A. lessonii *from Zanzibar at 5 m depth (circles), based on the physiological symbiont* (L*a*b* *color values, chlorophyll* a, *net photosynthesis) and holobiont variables (mortality, respiration, growth, motility) after exposure to: control/no stress (blue), a single stress event (green), episodic stress events (yellow) or chronic thermal stress (red).*

2.3.3 Identification of symbionts

Molecular fingerprinting of symbionts revealed that all the examined *A. gibbosa* specimens (n ≥ 17) contained the same diatom symbiont sequence, whereas *A. lessonii* (n ≥ 8) yielded seven haplotypes belonging to two lineages. The phylogenetic analysis suggests that all sequences can be attributed to diatoms of the order Fragilariales (Fig. 5). The remaining 13 sequences obtained from the isolates had non-algal affinities (Table S7).

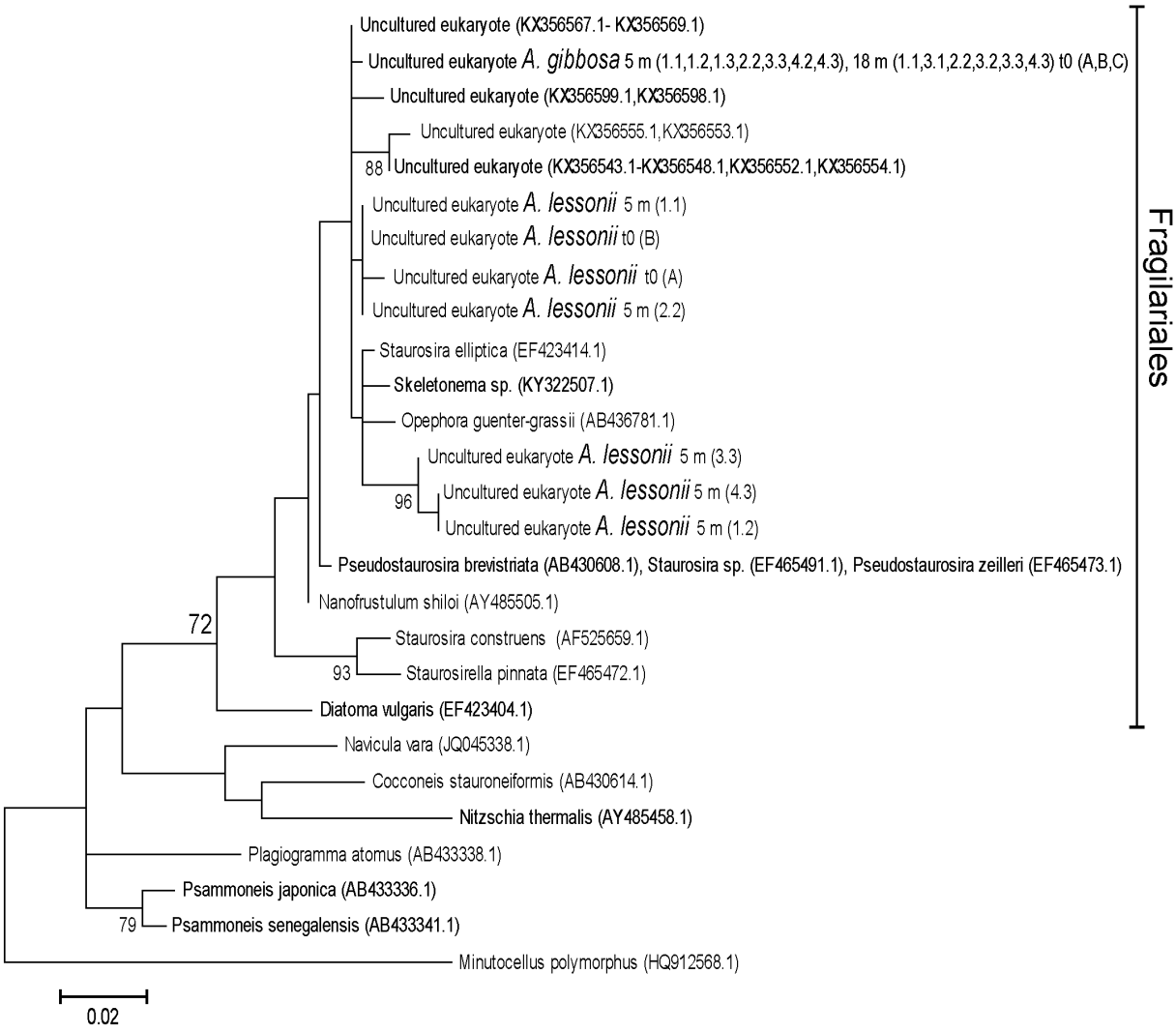

Figure 2-5. Phylogenetic assignment of diatom symbionts from Amphistegina gibbosa *from 18 m and 5 m depth in the Florida Keys, and from* A. lessonii *from Zanzibar in 5 m depth one week before the start of experimental treatment (t0) and after exposure to no stress/control (1.x), a single stress event (2.x), episodic stress events (3.x) or chronic thermal stress (4.x) as well as reference sequences of diatoms formerly described in association to large benthic foraminifera. Other taxa named 'uncultured eukaryote' refer to sequences from* A. lobifera *(Schmidt et al. 2016). Bootstrap support above 70% is given at the respective nodes. All recovered symbiont sequences were found within the Fragilariales with a bootstrap support of 72.*

All of the diatom sequences represent unsequenced diatom taxa, but they cluster together with diatom sequences obtained from the closely related *A. lobifera* (Schmidt et al. 2016a). Although the obtained sequences (442 bp) are too short for a taxonomic assignment to the species level, our results provide evidence for variability in the diatom assemblage of *A. lessonii*.

2.4 Discussion

Shells produced by members of the genus *Amphistegina* have been important constituents of reefal and carbonate-shelf sediments throughout most of the Cenozoic. How have these taxa been so consistently successful despite thermal maxima and glacial minima? Previous experimental studies have demonstrated similarities in growth rates and light tolerances of the widely distributed Indo-Pacific species, *A. lessonii,* and its western Atlantic-Caribbean sibling, *A. gibbosa* (e.g., Hallock et al. 1986; Walker et al. 2011). More recently, Prazeres et al. (2016, 2017b) reported that individuals of the shallowest-dwelling species, *A. lobifera*, show differences in resistance to elevated temperature and nutrients depending upon the habitats from which they are collected. Our study further explored these similarities and differences by comparing responses of *A. lessonii* and *A. gibbosa* to a suite of stresses that included single, episodic and chronic exposure to elevated temperature.

The responses of the three populations to chronic exposure to 32°C are consistent with bleaching. Both the type of response and the thermal threshold are similar to results from previous studies investigating diatom-bearing foraminifera (Talge and Hallock 2003; Schmidt et al. 2011, 2016a; Prazeres et al. 2016b). While the response to chronic thermal stress was similar across test populations, discriminant analysis revealed complete separation between *A. gibbosa* and *A. lessonii*, the latter being more resilient to all the tested stress scenarios. This difference is linked to higher diversity of diatom symbionts.

The responses to single and episodic stress events were generally minor and did not differ systematically among test groups. This confirms our prior observations regarding adaptation to episodic heat stress (Stuhr et al. 2017) and indicates that those observations apply to amphisteginids more broadly. Moreover, a diverse assemblage of shallow-water benthic foraminifera, including *Amphistegina* spp., was recently described from tropical tide pools with diurnal temperatures exceeding 35°C (Weinmann and Langer 2017), indicating that species of this genus are able to thrive in thermally variable habitats.

2.4.1 Inter-species variations

The systematic differences in thermal stress responses between *A. lessonii* and *A. gibbosa* holobionts were primarily seen in variables indicating symbiont performance such as Chl *a* concentrations and color measurements. While bleaching in response to chronic thermal stress diminished net photosynthesis in all test populations, it was predominantly manifested by higher (whiter) *L** and (greener) *a** values in *A. lessonii,* but by lower Chl *a* and (bluer) *b** values in *A. gibbosa* (Fig. 2). These dissimilar shifts might reflect the initially different colorations and may be related to differences in shell thickness (Hallock et al. 1986) or differences in photopigment compositions of the symbiont taxa.

While mortality was the variable that showed strongest inter-specific differences, those differences were likely artifacts of the timing of the collections and experiments. Mortality predominantly occurred in *A. gibbosa* in the control and single-stress treatments. The collection of *A. gibbosa* specimens and subsequent experiments took place during the time when field populations normally reproduce (Moses et al. 2017). Moreover, asexual reproduction often occurs in stock cultures during the first few weeks after collection (Hallock et al. 1995) and widespread gametogenesis was observed in *A. gibbosa* cultures within days after a summer power failure that lead to multiple-day heat exposure (Hallock, personal observation). We therefore conclude that the 'mortality' we observed in the *A. gibbosa* control and single-event treatments was likely caused by reproduction (Stuhr et al. 2017).

Average growth rates and motility were highest in *A. lessonii*. The two species were collected from geographically distant locations in different latitudinal ranges and hemispheres, so it was likely that their reproductive cycles were asynchronous. Offsets in ontogenetic phase would thus contribute to the detected species-specific responses in growth rates and mortality. Furthermore, both species were already above 0.7 mm in diameter at the start of the experiment and thus approaching the asymptote growth phase (e.g., Hallock et al. 1986), obstructing inter-species comparison.

The decrease in respiration rates in *A. lessonii* under chronic stress in contrast to elevated rates in *A. gibbosa* might be due to the non-linear relationship between respiration and temperature (Fujita et al. 2014). As oxygen consumption is lowest at optimum temperatures, the opposing trends might reflect different temperature range adaptations, with *A. lessonii* performing better at elevated temperatures. A higher thermal tolerance of this species is also suggested by overall higher motility and is in line with irradiance thresholds in *A. lessonii* compared to *A. gibbosa* (Hallock et al. 1986; Walker et al. 2011). Although additional conclusions can only be drawn after further investigations of the symbionts' ecology, the greater

resilience of the symbionts, resulting in less oxidative damage, likely can be attributed to the presence of different (and more diverse) symbionts in *A. lessonii*.

2.4.2 Intra-species variations

Despite the apparently homogeneous symbiont composition and stress-response patterns in both *A. gibbosa* test populations, we detected small differences in growth, photosynthesis and respiration rates. These were observed mostly in photosymbiont performance in the single-stress treatment. In the shallow-water population (Ag5), Chl *a* concentrations and net photosynthesis were not reduced by this treatment, while *b** values were slightly but insignificantly increased. Otherwise, the color values of both populations are remarkably similar. The same applies for holobiont variables. Collectively, these variations might indicate a slightly higher tolerance due to acclimatization to heat-stress events of specimens from the shallow-water site.

Water temperatures in the Florida Keys can fluctuate strongly close to the surface, as well as in deeper areas, though mean temperatures are higher in the shallow habitats (Leichter et al. 2006). Thus both test populations should have been similarly able to deal with thermally variable environments, but Ag5 could have been better acclimatized to thermal extremes. Similar response patterns to elevated temperatures were found in *A. lobifera* test populations collected from thermally quite different habitats in the Gulf of Aqaba and the Mediterranean, confirming that their thermal tolerance may be a conservative trait (Schmidt et al. 2016a). In comparison, intra-species sensitivity in *A. lobifera* on the Great Barrier Reef varied between habitats, suggesting a significant influence of environmental conditions on local acclimatization (Prazeres et al. 2016b). Overall, we infer that species-specific adaptive traits are likely the dominant determinant of the ecological ranges of *Amphistegina* spp., but further acclimatization to environmental conditions can contribute to higher temperature tolerance of local populations.

2.4.3 Symbiont assemblages

The species-specific differences in thermal stress response could potentially be the result of associations with different diatom species. We determined that *A. gibbosa* (from the Atlantic Ocean, Florida Keys) was associated with a single diatom sequence type, independent of sampling depth or exposure to stress, while *A. lessonii* (from the Indian Ocean, Zanzibar) bore more than one different but closely related diatom lineages. These results are in line with former genetic studies on diatom-bearing foraminifera (Holzmann et al. 2006; Barnes 2016; Schmidt et al. 2016a; Prazeres et al. 2017a), and also reflect the various degrees in flexibility and specificity described in the symbiosis between the dinoflagellate *Symbiodinium* and corals (reviewed by Baker 2003) or soritid foraminifera (Garcia-Cuetos et al. 2005; Pochon et al. 2007; Fay et al. 2009). Phylogenetic analyses of diatom sequences

from specimens of *A. gibbosa* hosts sampled over several years throughout various locations in the Florida Keys found only one dominant species (Barnes 2016). All extracted sequences were nearly identical and closely related to an unnamed Fragilariaceae diatom which has 13 substitutions compared to our sequences (GenBank Accession #JX413542.1 for 18S and JX413559.1 for rbcL). In summary, the symbiosis of *A. gibbosa* seems to be more restrictive (finical) than that of *A. lessonii*.

In contrast, the diatom sequences from *A. lessonii* support the hypothesis of higher plasticity in the symbiont assemblage of other amphisteginids (Schmidt et al. 2016a; Prazeres et al. 2017a). Similarly, investigations on the microbiome of *A. lobifera* collected from different habitats on the Great Barrier Reef, southwest Pacific, found these LBF to form persistent symbiosis, but with clear site-specific differences in the symbiont assemblages (Prazeres et al. 2017a).

Phylogenetic analyses of LBF belonging to the same clade as *Amphistegina* further support the findings that diatom-bearing LBF species prefer different symbiont types, but the species are flexible around this type (Holzmann et al. 2006). The extremely heat-tolerant species *Pararotalia calcariformata* was found to form a symbiosis with at least three different diatoms, but specifically with *Minutocellus polymorphus*, which is suspected to be responsible for its high thermal tolerance (Schmidt et al. 2015).

Species of dinoflagellate-bearing foraminifera also show a combination of host-symbiont specificity (Garcia-Cuetos et al. 2005) and flexibility (Pochon et al. 2007). Distinct distributions of different *Symbiodinium* types correlate geographically with environmental variables such as sea-surface temperature (Momigliano and Uthicke 2013), and are even found intracellularly in single specimens (Fay et al. 2009).

Jointly, these studies suggest that foraminifera-microalgae associations are species-specific but also vary within species in response to environmental factors. As these might substantially influence the ecological ranges and adaptive capacities of LBF, further investigations are crucial. These should additionally target the ecological preferences of the hosted diatoms.

2.4.4 Implications

Benthic foraminifera are among the most abundant photosymbiotic organisms in coral-reef ecosystems, but the drivers of their sensitivity to climate change-induced variations of ocean conditions remain poorly constrained. Considering the implications for paleoecology, geochemical cycles and the application of LBF as indicator taxa in reef management, intra- and inter-species variations in their responses to ocean warming are of major concern. Our experiment showed that

thermal stress responses can vary between species, though the effects of prior acclimatization (thermal history) were minor. Inter-species variations correlated with the detected variations in symbiont assemblages and corresponded to previous research on diatom-symbiont associations in relation to plasticity of holobiont physiology (Prazeres et al. 2016b, 2017a; Schmidt et al. 2016a). Further studies are imperative to determine how far these associations are shaped by environmental factors, and how variations in flexibility of symbiotic relationships contribute to resilience and adaptive capacity of photosymbiont-bearing foraminifera to changing environmental conditions. This needs to be done on a global scale, encompassing long-term monitoring, and include multi-parameter environmental factors.

2.5 Acknowledgements

This project was funded by the Leibniz Association (SAW-2014-ISAS-2) awarded to HW and JR. Sampling in the Florida Keys was conducted under the Research Permit No. FKNMS-2015-026. Samples from Zanzibar were collected under the Revolutionary Government of Zanzibar 434 (S.M.Z) Research Permit No. 0671.

2.6 Supplementary materials

*Table S2-1. P-values of two-sided F-Tests for variables CIE L*a*b* color space values, chlorophyll a concentrations (Chl a), oxygen production (net and gross photosynthesis) and oxygen consumptions rates (respiration), growth (increase in surface area), motility (movement within vials) and mortality (frequency of empty shells) of different test populations of* Amphistegina *species exposed to different thermal-stress treatments: μ_1 control, μ_2 = single stress events, μ_3 = episodic stress events, μ_4 = chronic stress. A closed testing procedure is used. P-values are marked* ***bold*** *(italic) if corresponding hypotheses are rejected at the* ***global*** *(only at the local) level of significance.*

Variable	H_0	*A. gibbosa*, 18 m	*A. gibbosa*, 5 m	*A. lessonii*, 5 m
L* value	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	0.082	0.098	**0.012**
	$\mu_1 = \mu_2 = \mu_3$	0.354	0.595	0.604
	$\mu_1 = \mu_2 = \mu_4$	0.094	0.108	**0.009**
	$\mu_1 = \mu_3 = \mu_4$	0.083	0.067	**0.043**
	$\mu_2 = \mu_3 = \mu_4$	0.375	0.217	**0.016**
	$\mu_1 = \mu_2$	0.483	0.799	0.956
	$\mu_1 = \mu_3$	0.312	0.537	0.632
	$\mu_1 = \mu_4$	0.110	0.065	*0.041*
	$\mu_2 = \mu_3$	0.940	0.852	0.638
	$\mu_2 = \mu_4$	0.502	0.296	*0.039*
	$\mu_3 = \mu_4$	0.427	0.203	*0.042*
a* value	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	**0.017**	**0.020**	**0.008**
	$\mu_1 = \mu_2 = \mu_3$	0.266	0.184	0.272
	$\mu_1 = \mu_2 = \mu_4$	**0.008**	0.052	**0.006**
	$\mu_1 = \mu_3 = \mu_4$	**0.018**	**0.014**	**0.034**
	$\mu_2 = \mu_3 = \mu_4$	0.293	0.115	**0.018**
	$\mu_1 = \mu_2$	*0.038*	0.614	0.629
	$\mu_1 = \mu_3$	0.271	0.129	0.338
	$\mu_1 = \mu_4$	**0.018**	0.059	*0.037*
	$\mu_2 = \mu_3$	0.576	0.528	0.543
	$\mu_2 = \mu_4$	0.316	0.231	*0.041*
	$\mu_3 = \mu_4$	0.158	0.239	0.060
b* value	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	**< 0.001**	**0.004**	**0.034**
	$\mu_1 = \mu_2 = \mu_3$	**0.009**	0.413	0.238
	$\mu_1 = \mu_2 = \mu_4$	**< 0.001**	**0.008**	0.065
	$\mu_1 = \mu_3 = \mu_4$	**0.001**	**0.021**	0.086
	$\mu_2 = \mu_3 = \mu_4$	**0.003**	**< 0.001**	0.068
	$\mu_1 = \mu_2$	0.150	0.646	0.869
	$\mu_1 = \mu_3$	**0.008**	0.506	0.327
	$\mu_1 = \mu_4$	**< 0.001**	*0.030*	0.168
	$\mu_2 = \mu_3$	0.431	*0.027*	0.268
	$\mu_2 = \mu_4$	**0.005**	**0.001**	0.151
	$\mu_3 = \mu_4$	**< 0.005**	**0.007**	0.369
Chlorophyll a	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	**< 0.001**	**< 0.001**	0.223
	$\mu_1 = \mu_2 = \mu_3$	**0.001**	**0.005**	0.854
	$\mu_1 = \mu_2 = \mu_4$	**< 0.001**	**0.001**	0.233
	$\mu_1 = \mu_3 = \mu_4$	**< 0.001**	**< 0.001**	0.337
	$\mu_2 = \mu_3 = \mu_4$	**0.003**	**0.001**	0.140

	$\mu_1 = \mu_2$	**0.011**	0.368	0.547
	$\mu_1 = \mu_3$	**0.003**	**0.008**	0.855
	$\mu_1 = \mu_4$	**0.002**	**0.005**	0.312
	$\mu_2 = \mu_3$	0.137	*0.044*	0.509
	$\mu_2 = \mu_4$	**0.017**	**0.012**	0.194
	$\mu_3 = \mu_4$	*0.031*	0.083	0.463
Net Photosynthesis[1]	$\mu_1 = \mu_2 = \mu_3 = \mu_4$		0.080	**0.045**
	$\mu_1 = \mu_2 = \mu_3$		0.113	0.189
	$\mu_1 = \mu_2 = \mu_4$	**0.001**	*0.003*	**0.020**
	$\mu_1 = \mu_3 = \mu_4$		0.150	0.125
	$\mu_2 = \mu_3 = \mu_4$		0.062	**0.012**
	$\mu_1 = \mu_2$	*0.039*	0.795	0.173
	$\mu_1 = \mu_3$		0.174	0.241
	$\mu_1 = \mu_4$	**0.007**	*0.017*	0.054
	$\mu_2 = \mu_3$		*0.025*	0.700
	$\mu_2 = \mu_4$	0.091	*0.005*	**0.023**
	$\mu_3 = \mu_4$		*< 0.001*	*0.026*
Gross Photosynthesis[1]	$\mu_1 = \mu_2 = \mu_3 = \mu_4$		0.837	**0.007**
	$\mu_1 = \mu_2 = \mu_3$		*0.018*	0.605
	$\mu_1 = \mu_2 = \mu_4$	**0.001**	0.550	**0.001**
	$\mu_1 = \mu_3 = \mu_4$		0.843	**0.029**
	$\mu_2 = \mu_3 = \mu_4$		*0.025*	**0.018**
	$\mu_1 = \mu_2$	0.064	*0.026*	0.313
	$\mu_1 = \mu_3$		*0.010*	0.654
	$\mu_1 = \mu_4$	**0.006**	0.767	**0.013**
	$\mu_2 = \mu_3$		0.967	0.694
	$\mu_2 = \mu_4$	0.068	*0.047*	**0.019**
	$\mu_3 = \mu_4$		*0.033*	**0.019**
Respiration[1]	$\mu_1 = \mu_2 = \mu_3 = \mu_4$		**0.035**	**0.016**
	$\mu_1 = \mu_2 = \mu_3$		0.462	0.106
	$\mu_1 = \mu_2 = \mu_4$	0.122	**0.017**	**0.028**
	$\mu_1 = \mu_3 = \mu_4$		**0.007**	**0.006**
	$\mu_2 = \mu_3 = \mu_4$		0.534	0.573
	$\mu_1 = \mu_2$	0.772	*0.045*	*0.029*
	$\mu_1 = \mu_3$		0.227	0.110
	$\mu_1 = \mu_4$	0.203	**0.006**	*0.029*
	$\mu_2 = \mu_3$		0.153	0.202
	$\mu_2 = \mu_4$	0.268	0.447	0.454
	$\mu_3 = \mu_4$		*0.024*	0.162
Growth	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	0.066	**0.002**	**0.012**
	$\mu_1 = \mu_2 = \mu_3$	0.366	0.079	0.314
	$\mu_1 = \mu_2 = \mu_4$	0.054	**0.005**	**0.041**
	$\mu_1 = \mu_3 = \mu_4$	0.144	**0.001**	**0.011**
	$\mu_2 = \mu_3 = \mu_4$	*< 0.001*	0.075	**0.001**
	$\mu_1 = \mu_2$	*0.004*	0.193	0.220
	$\mu_1 = \mu_3$	0.189	0.060	0.224
	$\mu_1 = \mu_4$	*0.002*	**0.003**	*0.036*
	$\mu_2 = \mu_3$	0.066	0.807	*0.020*

	$\mu_2 = \mu_4$	*< 0.001*	0.130	**0.003**
	$\mu_3 = \mu_4$	*0.003*	*0.049*	0.072
Motility	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	0.367	0.340	0.703
	$\mu_1 = \mu_2 = \mu_3$	*0.020*	0.231	0.096
	$\mu_1 = \mu_2 = \mu_4$	*0.042*	0.104	0.938
	$\mu_1 = \mu_3 = \mu_4$	0.495	0.361	0.485
	$\mu_2 = \mu_3 = \mu_4$	0.055	0.205	0.127
	$\mu_1 = \mu_2$	0.153	0.956	0.185
	$\mu_1 = \mu_3$	0.057	0.160	0.116
	$\mu_1 = \mu_4$	*0.019*	0.083	0.710
	$\mu_2 = \mu_3$	0.369	0.318	1.000
	$\mu_2 = \mu_4$	*0.005*	0.175	0.230
	$\mu_3 = \mu_4$	*0.006*	*0.003*	0.116
Mortality[2]	$\mu_1 = \mu_2 = \mu_3 = \mu_4$	**0.001**	**0.006**	1.000
	$\mu_1 = \mu_2 = \mu_3$	**0.040**	0.224	1.000
	$\mu_1 = \mu_2 = \mu_4$	**0.012**	**0.023**	1.000
	$\mu_1 = \mu_3 = \mu_4$		**0.014**	1.000
	$\mu_2 = \mu_3 = \mu_4$		**< 0.001**	1.000
	$\mu_1 = \mu_2$	0.267	0.133	1.000
	$\mu_1 = \mu_3$	**0.005**	0.130	1.000
	$\mu_1 = \mu_4$	**0.006**	**0.013**	1.000
	$\mu_2 = \mu_3$	**< 0.001**	*0.003*	1.000
	$\mu_2 = \mu_4$	**< 0.001**	**< 0.001**	1.000
	$\mu_3 = \mu_4$	1.000	0.233	1.000

[1] Values for the episodic-stress treatment (μ_3) are missing.
[2] Due to numerical problems (separation) null hypotheses $\mu_1 = \mu_3 = \mu_4$ and $\mu_2 = \mu_3 = \mu_4$ are not testable.

Table S2-2. Mortality of Amphistegina gibbosa *from 18 m and 5 m depth, Florida, and* A. lessonii *from 5 m depth in Zanzibar, Tanzania, exposed to different thermal-stress treatments estimated after every episodic stress event. Average frequency (percent) and SE of mortality (empty shells) per treatment (n = 3).*

Group	Treatment	3 days	12 days	21 days	30 days
***A. gibbosa*, 18 m**	Control	0.00 ± 0.00	0.13 ± 0.20	0.13 ± 0.20	0.33 ± 0.27
	Single	0.00 ± 0.00	0.07 ± 0.14	0.27 ± 0.26	0.53 ± 0.29
	Episodic	0.00 ± 0.00	0.00 ± 0.00	0.00± 0.00	0.00 ± 0.00
	Chronic	0.00 ± 0.00	0.00 ± 0.00	0.00± 0.00	0.00 ± 0.00
***A. gibbosa*, 5 m**	Control	0.00 ± 0.00	0.07 ± 0.14	0.13 ± 0.20	0.27 ± 0.26
	Single	0.00 ± 0.00	0.13 ± 0.20	0.33 ± 0.27	0.53 ± 0.29
	Episodic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.14
	Chronic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
***A. lessonii*, 5 m**	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Single	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Episodic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Chronic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

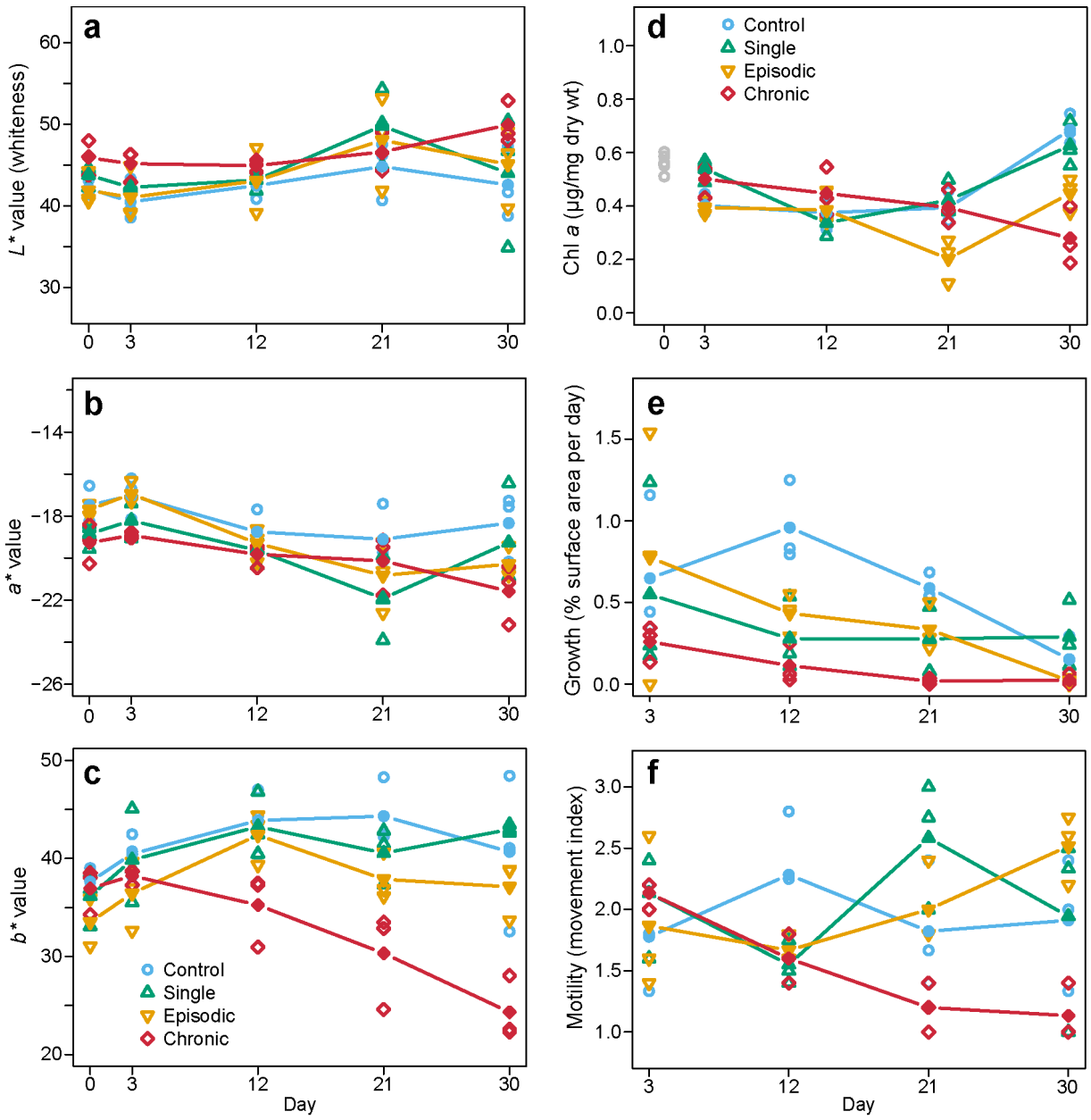

Figure S2-1. Repeated measurement of (a-c) CIE L*a*b* *color space values, (d) chlorophyll* a *concentrations, (e) growth rates, and (f) motility of* Amphistegina gibbosa *from 5 m over one month exposure to no stress/control (blue circles), a single stress event (green triangles), episodic stress events (yellow inverse triangles) or chronic thermal stress (red diamonds). Measurements were made after every episodic stress events and for CIE color space values and Chl* a *at the beginning of the experiment (day 0). Filled symbols connected by lines indicate the running means of each treatment (n = 3) on the respective sampling day. CIE space values indicate holobiont color measured on standardized images:* L* *= whiteness,* a* *= green (-) to magenta (+),* b* *= blue (-) to yellow (+), Chl* a *concentrations are normalized by dry weight, growth is measured from increase in surface are and motility estimated by amount of movement in the experimental vials. Equivalent results for* A. gibbosa *from 18 m are found in Stuhr et al. (2017).*

Table S2-3. Repeated measures ANOVA of Amphistegina gibbosa *from 5 m, Florida, exposed to different thermal-stress treatments. Results for the variables: motility, growth, CIE* L*a*b* *color space values and chlorophyll a concentrations, and different time periods.* P*-values <0.05 are printed in* ***bold****. Equivalent results for* A. gibbosa *from 18 m are found in Stuhr et al. (2017).*

Variable	Factor	df	F-ratio	*P*-value
*L** value	Treatment	3,8	1.01	0.436
	Time	3,24	7.43	**0.001**
	Treatment × Time	9,24	1.10	0.397
*a** value	Treatment	3,8	1.84	0.218
	Time	3,24	17.88	**< 0.001**
	Treatment × Time	9,24	2.23	0.056
*b** value	Treatment	3,8	7.70	**0.010**
	Time	3,24	7.45	**0.001**
	Treatment × Time	9,24	5.31	**0.001**
Chl *a*	Treatment	3,8	17.80	**0.001**
	Time	3,24	11.72	**< 0.001**
	Treatment × Time	9,24	7.50	**< 0.001**
Growth	Treatment	3,8	3.17	0.085
	Time	3,24	6.09	**0.003**
	Treatment × Time	9,24	1.37	0.257
Motility	Treatment	3,8	5.05	**0.030**
	Time	3,24	0.42	0.738
	Treatment × Time	9,24	2.78	**0.022**

*Table S2-4. Results of Tukey's HSD post hoc test (*A. gibbosa *from 5 m, Florida). All variables that showed significant interactions between Treatment × Time in the ANOVA (P-value < 0.05 in Table S2-3) were followed by Tukey's HSD test procedure to further identify difference between treatments (1 = control / no stress, 2 = single stress event, 3 = episodic stress events, 4 = chronic stress) within sampling days (time).*

Variable	Time (day)	Treatment	Estimate	SE	t-ratio	*P*-value
*a** value	30	1 - 4	3.244	1.059	3.06	0.029
*b** value	12	1 - 4	8.628	3.007	2.87	0.046
	21	1 - 4	13.992	3.007	4.65	0.001
		2 - 4	10.230	3.007	3.40	0.015
	30	1 - 4	16.356	3.007	5.44	< 0.001
		2 - 4	18.634	3.007	6.20	< 0.001
		3 - 4	12.781	3.007	4.25	0.003
Chl *a*	21	1 - 3	0.191	0.054	3.54	0.007
		2 - 3	0.219	0.054	4.05	0.002
		3 - 4	-0.191	0.054	-3.54	0.007
	30	1 - 3	0.238	0.054	4.40	0.001
		1 - 4	0.405	0.054	7.50	< 0.001
		2 - 3	0.179	0.054	3.31	0.013
		2 - 4	0.346	0.054	6.40	< 0.001
		3 - 4	0.167	0.054	3.10	0.021
Growth	12	1 - 2	0.680	0.245	2.78	0.045
		1 - 4	0.845	0.245	3.46	0.009
Motility	21	2 - 4	1.383	0.351	3.94	0.002
	30	3 - 4	1.383	0.351	3.94	0.002

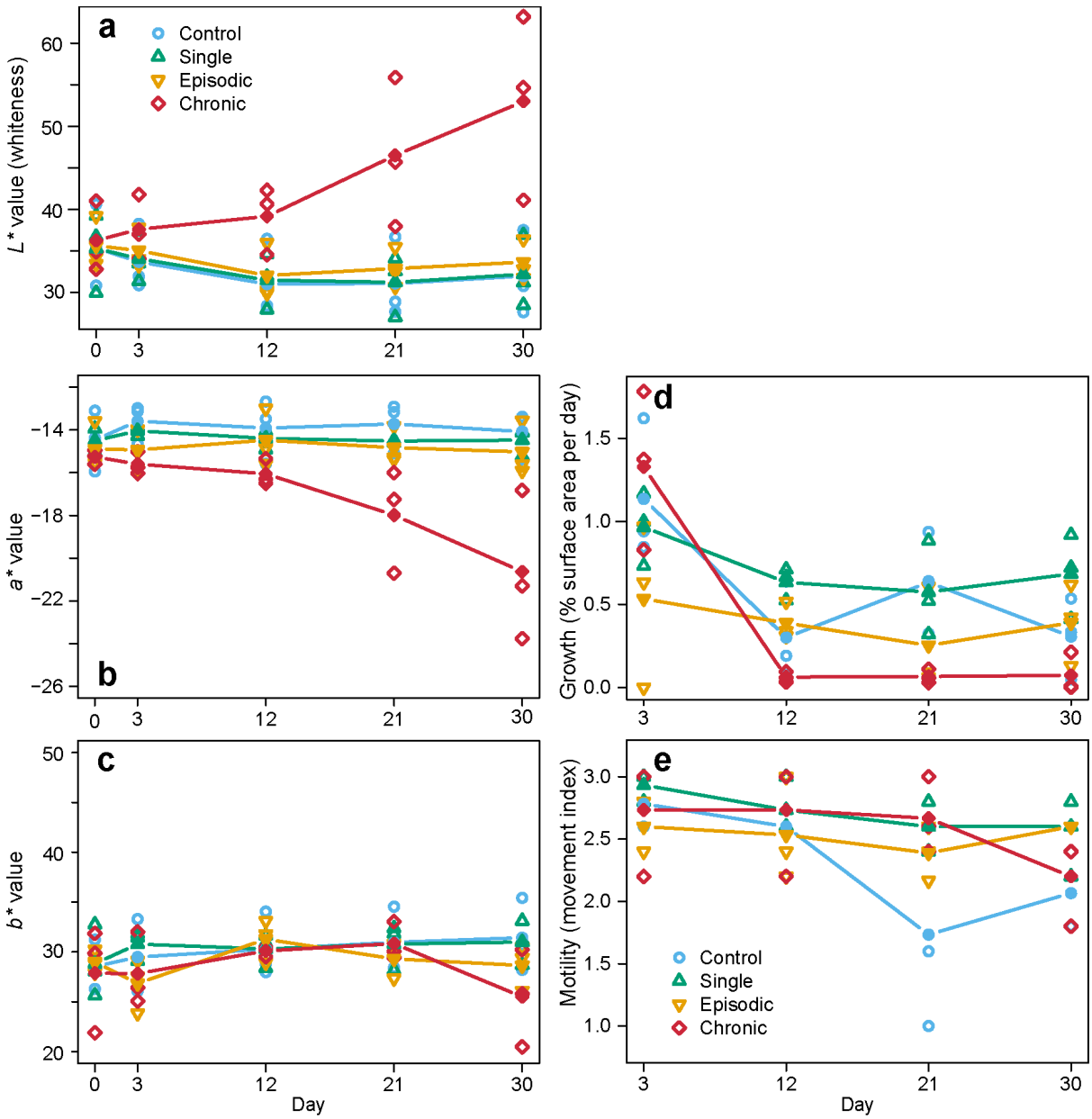

Figure S2-2. Repeated measurement of (a-c) CIE L*a*b* *color space values, (d) growth rates, and (e) motility of* Amphistegina lessonii *from 5 m over one month exposure to no stress/control (blue circles), a single stress event (green triangles), episodic stress events (yellow inverse triangles) or chronic thermal stress (red diamonds). Measurements were made after every episodic stress events and for CIE color space values at the beginning of the experiment (day 0). Filled symbols connected by lines indicate the running means of each treatment (n = 3) on the respective sampling day. CIE space values indicate holobiont color measured on standardized images:* L* *= whiteness,* a* *= green (-) to magenta (+),* b* *= blue (-) to yellow (+), growth is measured from increase in surface are and motility estimated by amount of movement in the experimental vials.*

Table S2-5. Repeated measures ANOVA of Amphistegina lessonii *from 5 m, Zanzibar, exposed to different thermal-stress treatments. Results for the variables: motility, growth, and CIE* L*a*b* *color space values and different time periods. P-values <0.05 are printed in* **bold**.

Variable	Factor	df	F-ratio	*P*-value
*L** value	Treatment	3,8	4.96	**0.031**
	Time	3,24	5.58	**0.005**
	Treatment × Time	9,24	6.44	**< 0.001**
*a** value	Treatment	3,8	6.13	0.424
	Time	3,24	7.64	**0.001**
	Treatment × Time	9,24	4.86	**0.001**
*b** value	Treatment	3,8	0.61	0.630
	Time	3,24	3.65	**0.027**
	Treatment × Time	9,24	2.47	**0.037**
Growth	Treatment	3,8	3.93	**0.054**
	Time	3,24	14.76	**< 0.001**
	Treatment × Time	9,24	2.51	**0.035**
Motility	Treatment	3,8	1.79	0.227
	Time	3,24	4.51	**0.012**
	Treatment × Time	9,24	1.43	0.231

*Table S2-6. Results of Tukey's HSD post hoc test (*Amphistegina lessonii, *5 m). All variables that showed significant interactions between Treatment × Time in the ANOVA (P-value < 0.05 in Table S2-5) were followed by Tukey's HSD test procedure to further identify difference between treatments (1 = control / no stress, 2 = single stress event, 3 = episodic stress events, 4 = chronic stress) within sampling days (time).*

Variable	Time (day)	Treatment	Estimate	SE	t-ratio	*P*-value
*L** value	21	1 - 4	-15.421	4.112	-3.75	0.013
		2 - 4	-15.283	4.112	-3.72	0.013
		3 - 4	-13.628	4.112	-3.31	0.027
	30	1 - 4	-21.020	4.112	-5.11	0.001
		2 - 4	-20.793	4.112	-5.06	0.001
		3 - 4	-19.343	4.112	-4.70	0.002
*a** value	21	1 - 4	4.264	1.127	3.78	0.009
		2 - 4	3.465	1.127	3.07	0.035
	30	1 - 4	6.555	1.127	5.81	< 0.001
		2 - 4	6.155	1.127	5.46	< 0.001
		3 - 4	5.604	1.127	4.97	0.001
Growth	3	3 - 4	-0.794	0.231	-3.44	0.009

Figure S2-3. Discriminant analysis including 95% ellipses for each population of Amphistegina gibbosa *from the Florida Keys at 18 m (square) and 5 m depth (diamond), and* A. lessonii *from Zanzibar at 5 m depth (circle), based on the physiological symbiont (CIE* L*a*b* *color space values, chlorophyll a concentration, net photosynthesis) and holobiont variables (mortality, respiration, growth, motility) after exposure to: control/no stress (blue), a single stress event (green), episodic stress events (yellow) or chronic thermal stress (red).*

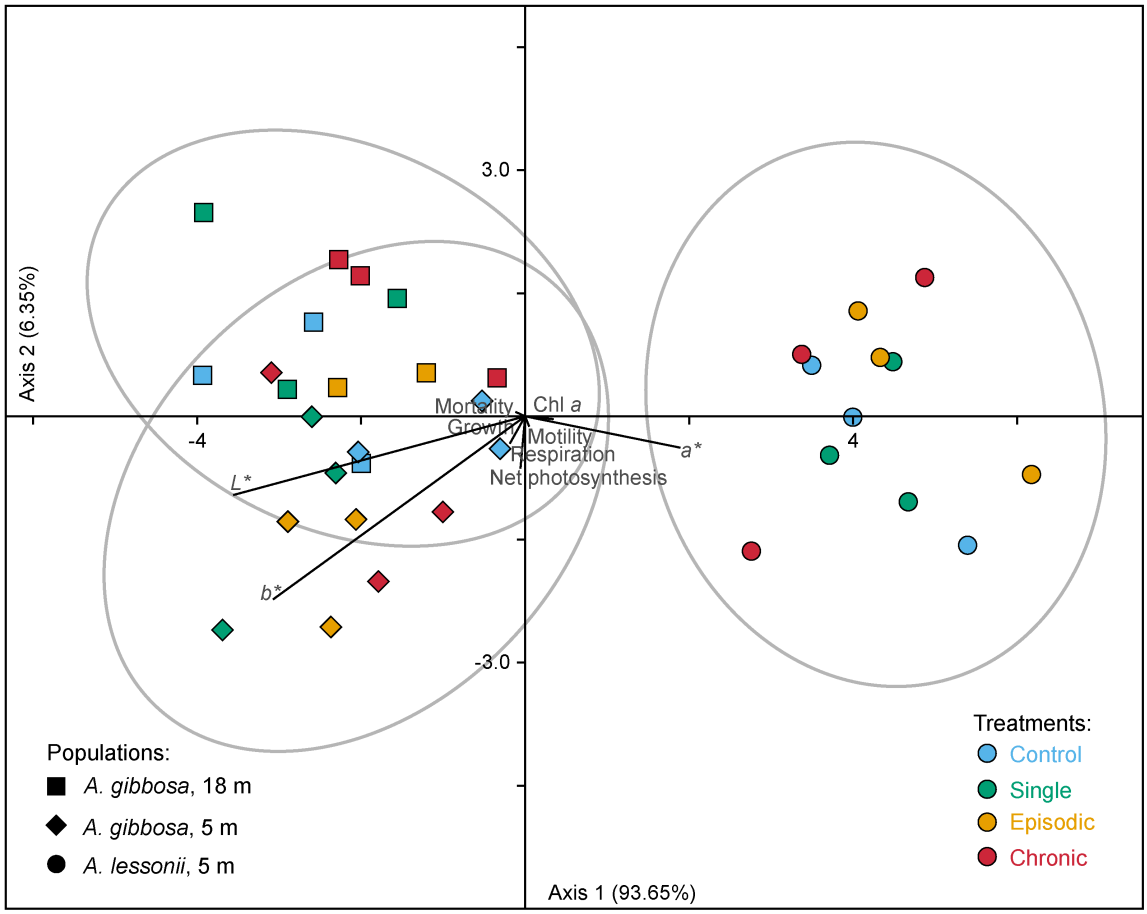

Table S2-7. Sequences considered as non-symbiont contaminants based on their BLAST result.

Sample	First BLAST hit
Ag_T0	Magnoliophyta; Liliopsida; *Najas flexilis*
Ag_T0_clone1	Eukaryota; Rhizaria; Cercozoa
Ag_T0_clone2	Alveolata; Euplotida; *Diophrys*
Ag_T0_clone3	Magnoliophyta; Liliopsida; *Najas flexilis*
Ag_T0_clone4	*Homo sapiens*
Ag 5 m 4.3_clone2	Uncultured peritrich ciliate (2d hit *Vaginicola* sp.)
Ag 18 m 4.3_clone3	Uncultured peritrich ciliate (2d hit *Vaginicola* sp.)
Ag 18 m 4.3_clone5	Uncultured peritrich ciliate (2d hit *Vaginicola* sp.)
Ag 18 m 2.2_clone1	Uncultured peritrich ciliate (2d hit *Vaginicola* sp.)
Ag 18 m 2.2_clone2	Uncultured peritrich ciliate (2d hit *Vaginicola* sp.)
Ag 18 m 3.3_clone3	Polychaeta; *Dinophilus*
Ag 18 m 4.3_clone3	Fungi; *Peniophora* sp. X-34
Ag 18 m 4.3_clone1	Eukaryota; Rhizaria; Cercozoa; *Bodomorpha* sp. Panama105

Chapter Three

REEF CALCIFIERS ARE ADAPTED TO EPISODIC HEAT STRESS BUT VULNERABLE TO SUSTAINED WARMING

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Personal contribution to chapter 3:

Idea and concept:
M. Stuhr developed the idea to focus on temporal variations in physiological responses to different thermal stress scenarios.

Research:
M. Stuhr carried out the experiment and conducted physiological sample analysis.
V. Rieder, J. Rahnenführer and M. Stuhr analyzed the data and created plots and tables.
M. Stuhr interpreted the data.

Writing and figure preparation:
M. Stuhr wrote the initial manuscript and prepared the figures, with contributions on method descriptions from V. Rieder.
M. Kucera, C.E. Reymond, P. Hallock, V. Rieder, and H. Westphal made improvements.

Abstract

Shallow marine ecosystems naturally experience fluctuating physicochemical conditions across spatial and temporal scales. Widespread coral-bleaching events, induced by prolonged heat stress, highlight the importance of how the duration and frequency of thermal stress influence the adaptive physiology of photosymbiotic calcifiers. Large benthic foraminifera harboring algal endosymbionts are major tropical carbonate producers and bioindicators of ecosystem health. Like corals, they are sensitive to thermal stress and bleach at temperatures temporarily occurring in their natural habitat and projected to happen more frequently. However, their thermal tolerance has been studied so far only by chronic exposure, so how they respond under more realistic episodic heat-event scenarios remains unknown. Here, we determined the physiological responses of Amphistegina gibbosa, an abundant western Atlantic foraminifera, to four different treatments—control, single, episodic, and chronic exposure to the same thermal stress (32°C)—in controlled laboratory cultures. Exposure to chronic thermal stress reduced motility and growth, while antioxidant capacity was elevated, and photosymbiont variables (coloration, oxygen-production rates, chlorophyll a concentration) indicated extensive bleaching. In contrast, single- and episodic-stress treatments were associated with higher motility and growth, while photosymbiont variables remained stable. The effects of single and episodic heat events were similar, except for the presumable occurrence of reproduction, which seemed to be suppressed by both episodic and chronic stress. The otherwise different responses between treatments with thermal fluctuations and chronic stress indicate adaptation to thermal peaks, but not to chronic exposure expected to ensue when baseline temperatures are elevated by climate change. This firstly implies that marine habitats with a history of fluctuating thermal stress potentially support resilient physiological mechanisms among photosymbiotic organisms. Secondly, there seem to be temporal constraints related to heat events among coral reef environments and reinforces the importance of temporal fluctuations in stress exposure in global-change studies and projections.

3.1 Introduction

The health and the geographical distribution of coral reefs are rapidly declining with ever increasing local and global pressures (Hoegh-Guldberg and Bruno 2010). Among the most prominent causes for this decline is long-term ocean warming, often manifested as transient heat events, which induce the loss of photosynthetic microalgae and/or photopigments from reef organisms, known as bleaching (Glynn 1996). The bleaching phenomenon was first observed among corals (Jaap 1979; Glynn 1996) and has since been documented among other photo-symbiotic tropical organisms including large benthic foraminifera (LBF) (Hallock et al. 1993). In recent years shallow-water tropical reef regions (e.g., the Great Barrier Reef) have undergone massive bleaching events (Hughes et al. 2017), which are expected to

become regular occurrences in the coming decade (Manzello 2015). The ongoing decline of coral populations and degradation of coral reefs has kindled interest in the thermal tolerance, adaptive value and stability of algal-invertebrate symbioses in these environments under higher temperature regimes (Middlebrook et al. 2008; Oliver and Palumbi 2011b).

The LBF *Amphistegina* spp. is a circumglobal, warm-water, calcifying eukaryote inhabiting oligotrophic coral-reef and shallow-shelf environments and hosting diatom photosymbionts (Hallock et al. 1986). Facilitated by their algal symbionts, LBF are vital constituents of coral-reef ecosystems (Doo et al. 2012a) and important marine calcifiers, responsible for the global production of approximately 0.1 Gt/year of carbonate sediments (Langer 2008). Due to their physiological sensitivity, LBF are commonly used as bioindicators for past and present environmental conditions such as water quality and coral reef health (Waters and Hallock; Hallock et al. 2003). The LBF are exceptionally useful models for studying the effects of global change on marine photosymbiotic calcifiers, primarily due to their abundance, fast growth, and easy handling in culture. Previous studies have shown that extreme and chronic thermal stresses have direct detrimental effects on calcification and overall host and photosymbiont (i.e., holobiont) fitness (Reymond et al. 2011; Schmidt et al. 2011; Sinutok et al. 2011; Uthicke et al. 2011). These studies have characterized either the immediate response to elevated temperatures or the effects of chronic exposure. Yet, how LBF react to episodic stress events, followed by intervals of thermal respite, is currently unknown. This is a vital aspect of adaptive physiology, because episodic stress followed by a phase of recovery, represents a realistic scenario for predicting the consequences of present and future global warming (Boyd et al. 2016).

Thermal stress appears to affect LBF primarily by impairing the function of the photosynthetic apparatus of the algal symbionts (Talge and Hallock 2003; Reymond et al. 2011; Schmidt et al. 2011). Such impairment can include reduced expression of the rate-limiting carbon-fixation enzyme RuBisCO (ribulose 1-5-biphosphate carboxylase/-oxygenase) (Doo et al. 2012b), reduced photopigment concentrations and photosynthetic performance (Reymond et al. 2011; Schmidt et al. 2011, 2014, 2016b; Sinutok et al. 2011; Uthicke et al. 2011) and reduced oxygen-production rates (Uthicke et al. 2011). Collectively, thermal stress can cause reduced growth, calcification, survivorship and fecundity (Reymond et al. 2011; Sinutok et al. 2011; Uthicke et al. 2011; Schmidt et al. 2014; Prazeres et al. 2016b, 2017b), as well as host inactivity (Schmidt et al. 2011). The exact kinds of molecular damage and cellular stress-related mechanisms that mediate these effects remain unknown. Similarly, the processes of recovery of LBF after stress exposure have not been previously reported. Recovery potential, however, is important in the context of episodic stress exposure, as such potential may facilitate survival despite peak

temperatures reaching the bleaching threshold, and could even increase thermal tolerance (Middlebrook et al. 2008; Oliver and Palumbi 2011b). Recovery responses could explain how LBF thrive in habitats where local temperatures can exceed temperatures that induce mortality when persistent over several days (Waters and Hallock).

The local effects of global warming include fluctuating physicochemical conditions across spatial and temporal scales (Vasseur et al. 2014; Boyd et al. 2016). In response to dynamic atmospheric and hydrographic processes, including cloud formation, wind-driven advection, diurnal heating and cooling, tides and internal waves, many abiotic parameters (e.g., intensity of solar irradiance, pH, temperature, and nutrient availability) can be altered on scales from hours to weeks (Putnam et al. 2010; Oliver and Palumbi 2011b; Mayfield et al. 2012; Vega-Rodriguez et al. 2015). Such fluctuations can be experienced from the surface of the ocean to mesophotic depths within coral-reef habitats (Leichter et al. 2006; Buerger et al. 2015). For example, the Florida Keys already experience high levels of thermal stress on a near-annual basis (Manzello 2015). Common daily subsurface temperature fluctuations here are on the order of 2 to 5°C (Leichter et al. 1996), but peak within-day ranges during summer can reach as much as 7 to 9°C at 20 to 30 meters depth, respectively (Leichter et al. 2006). Environmental heterogeneities influence the sensitivity of organisms to changing ocean conditions (Boyd et al. 2016) and should be considered when assessing their thresholds and tolerances. For instance, when temperature fluctuations are incorporated into model projections of global warming scenarios, the effects on species performance are stronger (Vasseur et al. 2014), highlighting the necessity to understand resilience to episodic stress events.

In this study, we investigated how the effects of episodic exposure to thermal stress, followed by recovery phases of thermal respite differ from the effects of chronic exposure to heat stress in LBF. Along the lines of earlier studies conducted on corals (Middlebrook et al. 2008; Putnam et al. 2010; Mayfield et al. 2012; Rodolfo-Metalpa et al. 2014), our hypothesis emphasizes the role of thermal variations on the physiological performance of LBF. Specifically, we carried out a laboratory-based culturing experiment, exposing the common western Atlantic LBF species *Amphistegina gibbosa* to one of four treatments, (a) control, (b) a single thermal-stress event, (c) episodic thermal-stress events or (d) chronic thermal stress. Our goals were to determine (i) whether single or episodic event exposure to thermal stress causes similar physiological response as chronic exposure, (ii) if the initial physiological response recovers after the stress is released and (iii) if acclimatization occurs to repeated short-term stress events.

3.2 Materials and methods

3.2.1 Sample collection and preparation

For this study *A. gibbosa* were collected from 18 m depth at Tennessee Reef in the Florida Keys (Fig. 3-1a), North Atlantic (24°45'8.33"N, 80°45'26.33"W), in June 2015. The site was previously described (Hallock et al. 1995; Manzello 2015) and sampling followed established protocols (Hallock et al. 1986). Sea-surface temperature (SST) in Florida ranges from winter minima of ~21°C to summer maxima of ~31.5°C with mean values of ~26.5°C (Leichter et al. 2006). *In situ* temperature measurements are slightly cooler than satellite-derived SST estimates (Vega-Rodriguez et al. 2015) and mean temperature decreases with depth (Leichter et al. 1996). The closest long-term monitoring station to our sampling site is at the Tennessee Reef Station managed by the Florida Keys National Marine Sanctuary (FKNMS) at 5 m depth (Fig. 3-1b) (Anderson et al. 2011). The temperature trends logged here reflect similar *in situ* bottom water temperatures, however unfortunately no long-term measurements are known for deeper areas but are assumed to be slightly cooler at 18 m depth.

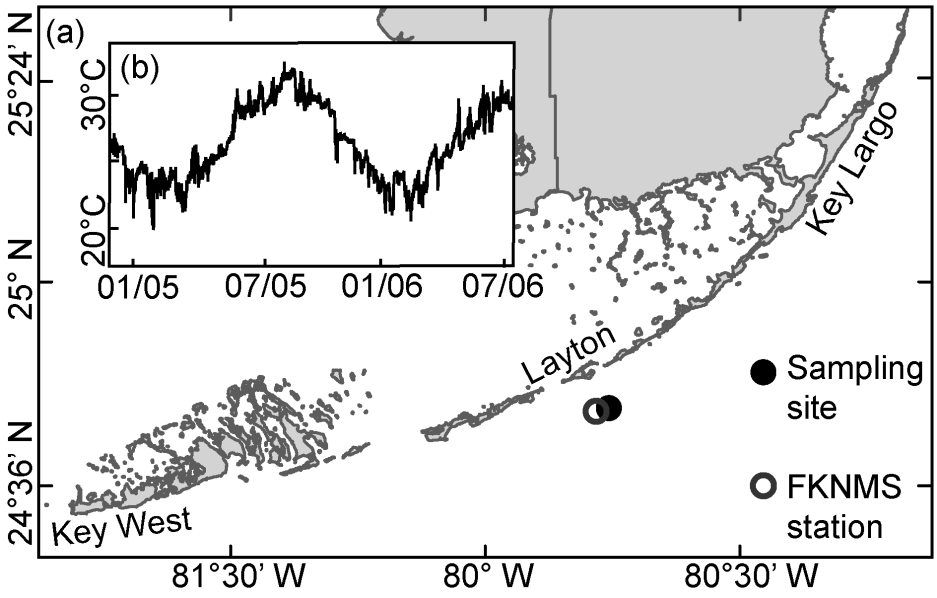

Figure 3-1. Map of the sampling location and local bottom water temperature measurements. (a) Map of the Florida Keys, USA, indicating the sampling site at Tennessee Reef in 18 m depth and the Florida Keys National Marine Sanctuary (FNKMS) station in 5 m depth where the bottom water temperature was continuously measured from 2004 to 2006; (b) Bottom water temperature at FKNMS station Tennessee Reef shows temperature fluctuations from 20°C in winter to above 30°C in summer (Anderson et al. 2011).

After sample preparation at the Keys Marine Laboratory (KML, Layton, USA) and sorting species in the laboratory of the University of South Florida (USF, St. Petersburg, USA), specimens were shipped (inside insulated containers for <24 h) to aquaria in Bremen, Germany. During preparation and transportation, temperature loggers (Hobo, Onset, USA) recorded an average temperature of 24.99 ± 0.27°C. All specimens were acclimated to 25.5 ± 0.5°C under 5–10 µmol photons m^{-2} s^{-1} on a 12-h light/dark cycle for three weeks prior to the initiation of the experiment, approximating mean baseline temperature appraised for their natural habitat at 18 m depth and 'stock-culture' conditions (Talge and Hallock 2003). Synthetic seawater (Tropic Marine Sea Salt, Germany) was used and maintained at a salinity of 35.5, all specimens were fed 15 µl of autoclaved microalgae (Schmidt et al. 2016b) every nine days.

3.2.2 Mimicking thermal stress

Our experiment utilized a setup modified from (Schmidt et al. 2016b) that incorporated 12 independent aquaria (working volume 18 l). Previous experiments with *Amphistegina* spp. in culture have shown that large water volume and water movement are beneficial for extended maintenance, most likely as such setup mimics natural conditions better than small-volume stationary culture dishes (Hallock et al. 1986). For each experimental scenario, three randomly allocated replicate aquaria were used containing ~80 individuals of *A. gibbosa*. Each aquarium was equipped with a temperature sensor, titanium heating rod, and an aquarium pump (Nanoprop 5000, Aqua Medic, Germany). Temperature was controlled automatically with an AT-Control system (Aqua Medic, Germany) and logged with a HOBO data logger (Model UA-002-64, Pendant, Onset, USA) per treatment. Salinity, pH, and temperature were measured every second day in all aquaria, using a multimeter (WTW, Germany). Experimental light levels were set to ~5–10 µmol photons m^{-2} s^{-1} on a 12-h light/dark cycle, supplied by tri-chromatic daylight fluorescent glow tubes (T5 Reef White 10K 54W, Aqua Medic, Germany) and regulated by a light controller unit (Aqua Medic, Germany). Light levels inside the aquaria were recorded at the beginning and the end of the experiment using a light quantum meter (LI-COR LI-250A) with a submersible micro-quantum sensor (Waltz, Germany). Within each aquarium, several subsets of foraminifers were kept in glass vials, covered with a 400 µm nylon mesh to allow the water to circulate into the vial but keep the specimens in their vials throughout the experiment.

With the expectation that the changes in photosymbiont- and holobiont-specific response variables can provide insights into the interactions between the symbiotic partners over time and after repeated exposure, we monitored them regularly throughout the experiment (Fig. 3-2). The photosymbiont parameters assessed included photopigment concentration, bleaching frequency, photosynthetic rates and changes in coloration. The impact on overall fitness of the LBF, the onset of

oxidative stress on the holobiont level and disruption in calcification, were assessed by motility, mortality, antioxidant capacity against peroxyl radicals (ACAP), respiration and growth rates. A fixed subset of five *A. gibbosa* specimens from each aquarium was used to record these variables the day before the experiment and after each episodic temperature stress event, which occurred (i.e., on days 0, 3, 12, 21 and 30). Over three days the temperature peaks were simulated by slowly raising the temperature by ~0.25°C per hour for the first 24 h, for the second day keeping it at 32°C and on the third day, slowly letting it drop back to control temperature of 25.5°C. In the chronic stress treatment, the temperature was raised similarly in the first day of the experiment, but kept at 32 ± 0.5°C until the end of the experiment. Specimens that appeared dead or experienced mechanical damage during the experiment were excluded from further data analysis, but remained within the experiment. For Chl *a* and ACAP analyses, separate subsamples were taken from all aquaria on each sampling day and immediately frozen at –80°C until further processing.

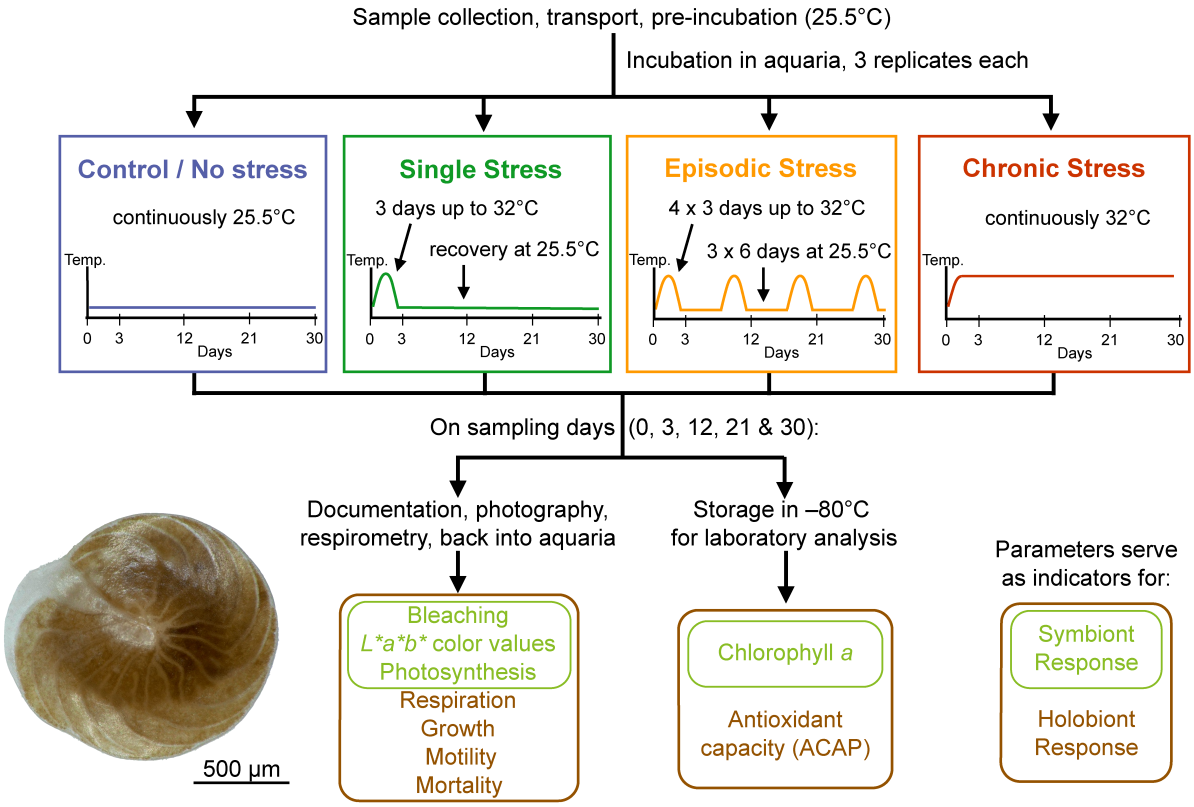

Figure 3-2. Flowchart illustrating the experimental setup including the four treatments and measured variables. Each experimental variable is shown as either indicating the symbiont or the holobiont response. The photo in the lower left corner shows a photo of an adult Amphistegina gibbosa *specimen, taken with a digital microscope (VHX-5000, Keyence, Germany).*

3.2.3 Motility and growth

Motility is an indicator for the foraminifers' activity and fitness (Schmidt et al. 2011). By means of their reticulopodial network, the specimens were able to climb the walls of the glass vial and attach to the mesh covering the vial. On each sampling day, the location of all specimens within the glass vial was documented and rated by the distance they moved since the previous sampling: on the bottom '1 = Low', on the wall '2 = Medium' or on the mesh '3 = High'. The average motility in each vial was estimated by the following equation:

$$\text{Motility index} = \frac{(1 \cdot N_{bottom} + 2 \cdot N_{wall} + 3 \cdot N_{mesh})}{N_{total}} \qquad N = \text{number of specimens.}$$

To determine the growth rates and coloration, high-resolution photographs were taken on a standardized color background (RAL 4007-P) using fixed settings and stable light conditions with a Zeiss Discovery V8 SteREO Microscope connected to a Canon EOS 600D camera. The surface area of each specimen was measured in photographs via the software Fiji v2.0.0 (Schindelin et al. 2012). A precision of 1% was predetermined for this method by repeated measurements of five specimens 20 times. Growth was estimated as an increase in cross-sectional surface area (mm^{-2}) of all surviving foraminifera in comparison to the previous measurement and calculated as growth per day (% d^{-1}) (ter Kuile and Erez 1984).

3.2.4 Mortality and bleaching

As described by (Bernhard 2000), empty shells reflect mortality, which is either due to stress-induced death, natural causes (old age), or reproduction. Our sampling strategy and the contorted form of the vials inhibited detection of any juveniles, which resulted from sexual or asexual reproduction. The outermost (newest) chamber normally lacks symbionts (see image in Fig.3-2). When any older chambers were pale or showed white spots, these specimens were recorded as mottled or partly bleached (Talge and Hallock 2003). The means and SE of the proportions (n = 3) of mortality as well as partial bleaching (mottling) of the surviving specimens out of the 5 initially pooled individuals were calculated.

3.2.5 Holobiont color

The holobiont color was determined using the CIE *L*a*b** color space values of each foraminifer after Hosono et al. (Hosono et al. 2012). In each image, holobiont color and background color were transformed into CIE *L*a*b* color space by using the color space converter in Fiji (Schwartzwald 2012) and determined with the same software. Artifacts of light reflecting on the shiny foraminifera shells were excluded. Mean color values were corrected by the color determined for the standardized color pallet in the image (*L** = 30.24, *a** = 12.25, *b** = -5.47) (Hosono et al. 2012). The resulting color values represent the three coordinates within the CIE *L*a*b**

color space: *L** indicates whiteness (brightness) of the color (0 = black, 100 = white), *a** indicates the position between green (negative values) and magenta (positive values), and *b** indicates the position between blue (negative values) and yellow (positive values).

3.2.6 Respiration and net photosynthesis

Respiration was determined by measuring the oxygen concentration in custom-made (~1 ml) respirometry chambers with 400–600 µm diameter oxygen micro-sensors (OX-MR, Unisense, Denmark). During the measurements, each chamber housed the fixed subset of five *A. gibbosa* and one chamber with only seawater served as a control for background respiration. The same specimens were used for each repeated measurement throughout the experiment. Micro-sensors were introduced into the airtight glass vials containing a magnetic stirrer and the foraminifers, separated by a mesh net. To keep the water temperature stable within the vials, they were submerged in a temperature-controlled water bath set to 25.5°C except for the chronic treatments specimens, for which the temperature was set to 32°C. After a dark acclimation phase of 45 min, respiration was determined during a 30-min dark phase, followed by a 30-min light phase for net photosynthesis measurements (adapted from Schmidt et al. 2011; Reymond et al. 2013; Fujita et al. 2014). During light incubations, light intensities replicated the conditions used during the experiment (5–10 µmol photons m^{-2} s^{-1}). Respiration (oxygen consumption) and net photosynthesis (oxygen production) rates were normalized to total surface area of the living foraminiferal specimens predetermined from photographs (as described in the previous section). The daily rates were extrapolated according to a 12-h day/night cycle and gross photosynthesis rates of each replicate were calculated by subtracting respiration from net photosynthesis rates.

3.2.7 Chlorophyll *a* concentration

To determine the photosymbiont biomass, the concentration of Chl *a* was measured, adapted from Schmidt et al. (Schmidt et al. 2011) by changing the protocol from using the foraminifers' wet weight to measuring the dry weight of the crushed specimens after extracting the pigment, reducing the potential risk of overestimating weight due to additional water. The remaining foraminiferal pellets were dried for at least 24 h at 40°C within Eppendorf vials and weighed to 0.001 mg accuracy. The resulting Chl *a* concentrations were normalized by the pellet dry weights

3.2.8 Antioxidant capacity against peroxyl radicals (ACAP)

From each aquarium, a subsample of 10 specimens was collected on each sampling day and immediately frozen (–80°C) for ACAP analysis. To evaluate the biological resistance of the LBF to peroxyl radicals, ACAP assays were performed to predict

the oxyradical-mediated effect on the physiological condition of organisms. Analyses utilized a fluorescence technique (Amado et al. 2009) and adapted for foraminiferal samples (Prazeres et al. 2016b). This method determines the antioxidant capacity indirectly by measuring the reactive oxygen species (ROS) concentration in each sample. Thus, a high capacity to neutralize peroxyl radicals results in low ROS concentrations, indicating a high ACAP. The antioxidant capacity was calculated according to Amado et al. (Amado et al. 2009) and is expressed as the inverse of the relative area (Prazeres et al. 2016b).

3.2.9 Data analysis

A principal component analysis (PCA based on correlation matrix) of the variables bleaching, color values *L**, *a**, *b**, Chl *a*, ACAP, growth, motility, and frequency of empty shells on days 12, 21 and 30 was conducted using Past v3.11 (Hammer et al. 2001). All further statistical analyses were performed and figures were produced with the statistical programming software R (R Core Team 2016), Version 3.2.4.
Repeated-measures analysis of variance (ANOVA) for related (dependent) groups of aquaria was applied for growth, motility, net photosynthesis, respiration, Chl *a*, and ACAP, to test for differences between treatments using the R function `aov` to fit an analysis of variance model (Faraway 2016). In this model, aquarium as the blocking random factor was nested within treatment. Time (sampling day) and treatment were within and between fixed factors, respectively. With respect to the use of ANOVA, although the data distribution appeared to be normally distributed, the normality assumption could not be formally tested due to the small sample size in each aquarium. The small sample size also precluded the use of non-parametric methods.

For the binary outcomes of bleaching (mottling) and mortality, logistic regression with a random effect for aquaria was used by employing `glmer` in the R package `lme4` (Bates et al. 2015). By means of a likelihood ratio test, significant differences among treatments, times and the interaction between treatment and time can be tested, but due to numerical problems (quasi-complete separation), only descriptive analyses were conducted for bleaching and mortality.

Missing values due to high mortality in some replicates caused an unbalanced design. When there were a few missing values, they were imputed using the R package longitudinalData (function imputation with method linearInterpol.locf (Genolini 2016)) to conduct an ANOVA. Intermediate missing values were linearly interpolated whereas last observation carried forward (locf) and next observation carried backward (nocb) imputations were used for monotonic missing values (at the start and the end of the trajectories). In the case of oxygen consumption (respiration) and oxygen production (net photosynthesis), all values are missing for episodic stress at day 30 due to instrument failure. To avoid the loss of one level

each in treatment and time, two analyses were performed on the datasets, the first without episodic stress and the second without day 30. To further identify individual differences between treatments within sampling times, significant ANOVAs were followed by Tukey's HSD test procedure using the R package lsmeans (Lenth 2016) thereby controlling for the multiple testing problem. All experimental data will be made available through the PANGAEA data repository.

3.3 Results

With respect to all photosymbiont-specific variables, the population exposed to chronic thermal stress showed the strongest response, including the highest frequency of bleaching and color changes, as well as the lowest Chl *a* concentration and oxygen-production rate at the end of the experiment (Tables 3-1 and 3-2, Table S3-1, Figs. 3-3 and 3-4b). The strength of the reaction increased with time. In contrast, the control treatment showed the least bleaching response, while oxygen production (net photosynthesis) increased, which is consistent with an increase in Chl *a* and colors over time (Table 3-1, Figs. 3-3 and 3-4b, Fig. S3-2). The responses in treatments with single and episodic stress were remarkably similar with respect to all photosymbiont-specific parameters and remained stable through the duration of the experiment (Table 3-2, Figs. 3-3 and 3-4b).

Table 3-1. Bleaching frequency of A. gibbosa *exposed to different thermal-stress treatments. Average frequency and SE (n = 3) of the proportion of partial bleaching (mottling) of surviving specimens (see mortality in Table 3-3) out of 5 pooled individuals in response to exposure to the different treatments: control / no stress, a single stress event, episodic stress events or chronic stress, over different time periods.*

Treatment	3 days	12 days	21 days	30 days
Control	0.07 ± 0.14	0.07 ± 0.14	0.13 ± 0.20	0.11 ± 0.18
Single	0.00 ± 0.00	0.35 ± 0.28	0.28 ± 0.26	0.33 ± 0.33
Episodic	0.07 ± 0.14	0.20 ± 0.23	0.27 ± 0.26	0.36 ± 0.28
Chronic	0.07 ± 0.14	0.22 ± 0.24	0.58 ± 0.28	0.93 ± 0.14

Table 3-2. Repeated measures ANOVA of A. gibbosa *exposed to different thermal-stress treatments. Results for the variables: motility, growth, CIE* L*a*b* *color space values, respiration, net photosynthesis, Chl* a*, and antioxidant capacity against peroxyl radicals (ACAP) and different time periods. P-values <0.05 are printed in bold. The results of Tukey's HSD post hoc test for all variables that showed significant interactions between Treatment × Time that further identifies individual differences between treatments at each sampling day are found in Table S3-1.*

Variable	Factor	df	F-ratio	*P*-value
*L**	Treatment	3,8	0.48	0.707
	Time	4,32	3.82	0.023
	Treatment × Time	12,32	2.57	0.031
*a**	Treatment	3,8	1.04	0.424
	Time	4,32	34.2	< 0.001
	Treatment × Time	12,32	4.88	0.001
*b**	Treatment	3,8	56.6	< 0.001
	Time	4,32	8.71	< 0.001
	Treatment × Time	12,32	10.5	< 0.001
Chl *a*	Treatment	3,8	47.2	< 0.001
	Time	3,24	4.69	0.010
	Treatment × Time	9,24	4.20	0.002
Net photosynthesis[a]	Treatment	2,6	1.64	0.270
	Time	3,18	0.45	0.721
	Treatment × Time	6,18	10.2	< 0.001
Net photosynthesis[b]	Treatment	3,8	0.12	0.944
	Time	2,16	0.85	0.445
	Treatment × Time	6,16	4.83	0.005
Respiration[a]	Treatment	2,6	2.32	0.180
	Time	3,18	1.14	0.358
	Treatment × Time	6,18	3.09	0.029
Respiration[b]	Treatment	3,8	1.28	0.344
	Time	2,16	0.73	0.498
	Treatment × Time	6,16	3.39	0.024
ACAP	Treatment	3,8	3.04	0.093
	Time	3,24	1.55	0.229
	Treatment × Time	9,24	4.77	0.001
Growth	Treatment	3,8	5.05	0.029
	Time	3,24	3.92	0.021
	Treatment × Time	9,24	0.71	0.691
Motility	Treatment	3,8	6.85	0.013
	Time	3,24	0.12	0.945
	Treatment × Time	9,24	5.49	< 0.001

[a] ANOVA was performed only for control, single- and chronic-stress treatments on all sampling times.

[b] ANOVA was performed for all treatments but only on sampling times 3, 12 and 21.

The reduced performance of the photosymbionts consistently seen in the chronic exposure treatment was mirrored by all holobiont variables. The chronic treatment showed the lowest growth rates and motility as well as the highest ACAP (Table 3-2, Table S3-1, Figs. 3-4c-e). These variables displayed a clear temporal trend. Oxygen consumption (respiration) showed no significant differences among the treatments and no trend (Fig. 3-4f). As in the photosymbiont-specific variables, the holobiont response appeared to be similar for the single- and episodic-stress treatments (Table 3-2, Figs. 3-4c-e). In most variables, the response of these treatments was comparable to the control, except for motility, which was significantly higher in the single- and episodic-stress treatments, and the ACAP, which showed different temporal trends. The single-stress treatment and the control also displayed similar mortality trends (Table 3-3, Fig. S3-1). Because empty shells were associated with treatments where photosymbiont and holobiont variables indicated optimum growth, and because the experiment was conducted at the time of year when these populations normally reproduce (Hallock et al. 1995), the presence of empty shells in this study is interpreted as being caused by reproduction. Because the interpretation of mortality as reproduction could not be assured and quantified, it has to be treated with caution. Since most standard errors are high, trends in the running mean should not be over interpreted.

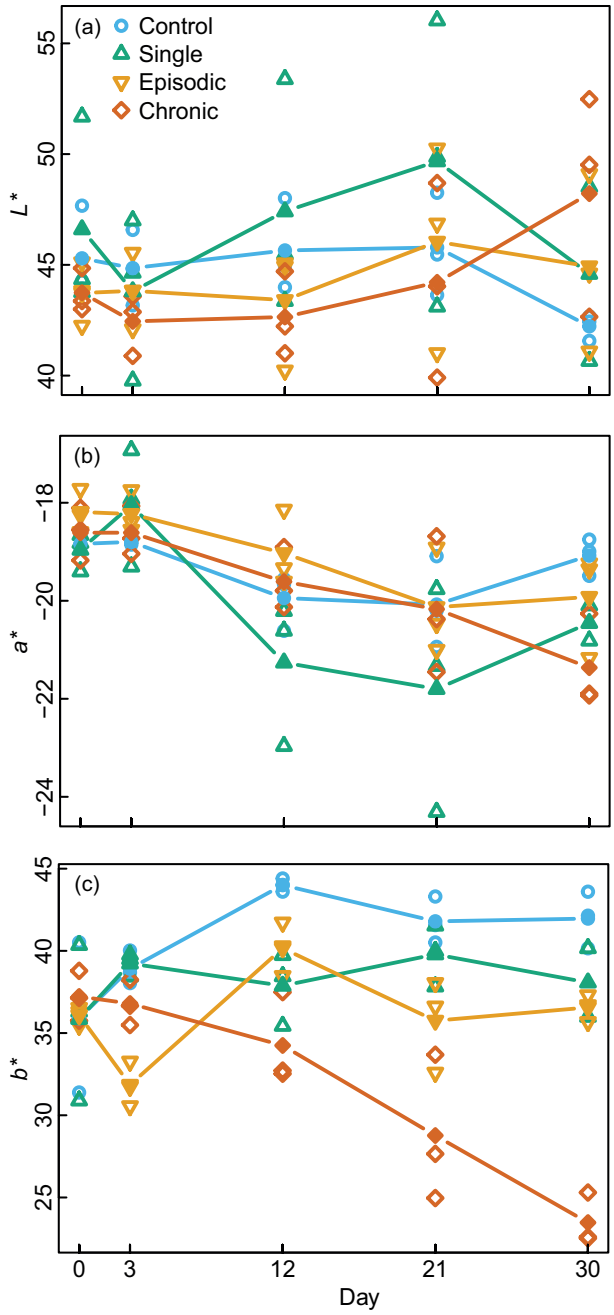

Figure 3-3. Repeated measurement of color values on A. gibbosa *in response to different thermal-stress treatments. The CIE color space values (a)* L* *= whiteness, (b)* a* *= green (-) to magenta (+), (c)* b* *= blue (-) to yellow (+) at time zero and subsequently after each episodic thermal stress event. The different treatments are depicted according to: control / no stress (blue circles), single stress event (green triangles), episodic stress events (yellow inverse triangles) and chronic stress (red diamonds). Filled symbols connected by lines represent the running means of each treatment (n = 3) on the respective sampling time (day).*

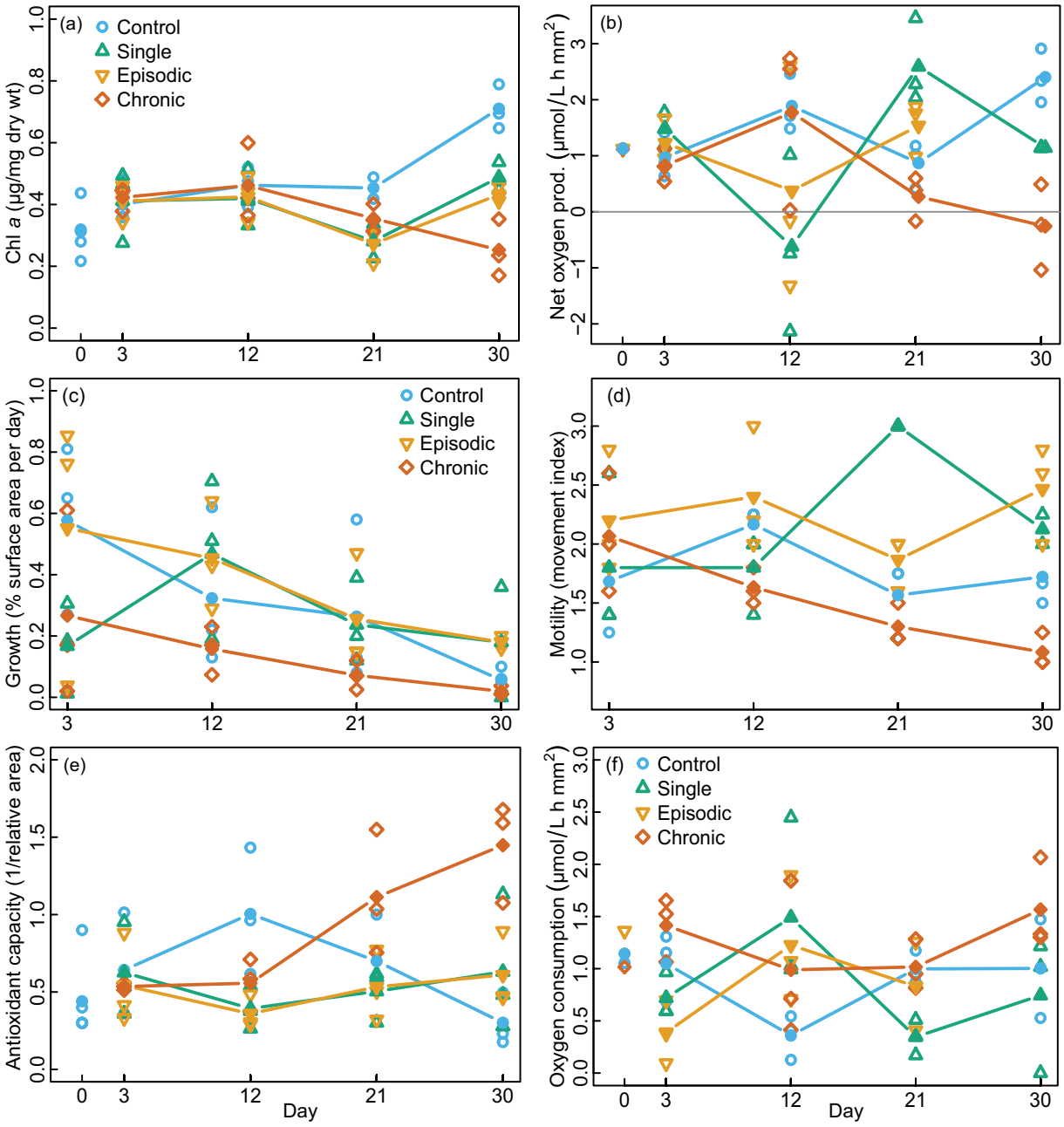

Figure 3-4. Repeated measurement of physiological variables on A. gibbosa *in response to different thermal-stress treatments. (a) Chl a concentration (µg per mg dry wt), (b) net photosynthesis (expressed as oxygen-production rate per surface area), (c) growth rates (as a percentage of increase in surface area per day since the previous sampling time), (d) motility index indicating the amount of movement within the experimental vials, (e) total antioxidant capacity against peroxyl radicals (ACAP, expressed as the inverse of the relative area of fluorescence produced by reactive oxygen species), (f) respiration (expressed as oxygen-consumption rate per surface area) at time zero and subsequently after each episodic thermal-stress event. The different treatments are depicted according to: control / no stress (blue circles), single stress event (green triangles), episodic stress events (yellow inverse triangles) and chronic stress (red diamonds). Filled symbols connected by lines represent the running means of each treatment (n = 3) on the respective sampling time (day).*

Table 3-3. Mortality of A. gibbosa *exposed to different thermal-stress treatments. Average frequency and SE (n = 3) of the proportion of mortality (empty shells) out of 5 pooled individuals in response to exposure to the different treatments: control / no stress, a single stress event, episodic stress events or chronic stress, over different time periods.*

Treatment	3 days	12 days	21 days	30 days
Control	0.00 ± 0.00	0.13 ± 0.20	0.13 ± 0.20	0.33 ± 0.27
Single	0.00 ± 0.00	0.07 ± 0.14	0.27 ± 0.26	0.53 ± 0.29
Episodic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Chronic	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

The overall pattern of stress response among the treatments through time is visualized by a principal component analysis (PCA, Fig. 3-5). The PCA portrays nine variables from day 12 to day 30 and reveals high collinearity in the response variables reflected in the fact that the first two principal components account for >60% of total variance. The biplot of these two principal components highlights the fundamental differences in directional response of the chronic treatment, which shows a stronger response with time, associated with ACAP and variables describing bleaching. In contrast, the single treatment and the episodic treatment appear to behave in a similar way. They show no unidirectional trend in their response and deviate from the control only due to higher motility and no significant increase in Chl *a* through time. The biplot also illustrates the consistency among the replicates of the treatments, which seems to show decreasing variance through time. The larger fluctuations in the response through time observed in the single-stress treatment have to be seen in the light of the highest frequency of empty shells (mortality most likely due to reproduction) in this treatment, implying that a large part of this variability could be due to high motility and color changes prior to reproduction.

3.4 Discussion

The results demonstrate that the physiological effects of single and episodic stress events on photosymbiotic calcifiers are markedly different compared to chronic stress. Single and episodic thermal peaks did not impair the function of *A. gibbosa*, while chronic stress damaged the algal photosymbionts, induced an antioxidant defense response, and compromised the overall holobiont health and activity. The divergence in physiological responses between the chronic and episodic thermal stress seems to have developed between day 3 and day 12 of the experiment (Figs. 3-3 and 3-4). This divergence emphasizes not only the temporal tipping point and damage associated with chronic stress but also the importance of respite phases during thermal stress. The temperature conditions in this experiment emulate water temperature variability and duration (hours to days) shifts >5°C recorded in tropical reefs (Leichter et al. 2006; Boyd et al. 2016) and therefore present real-life scenarios of temperature stress.

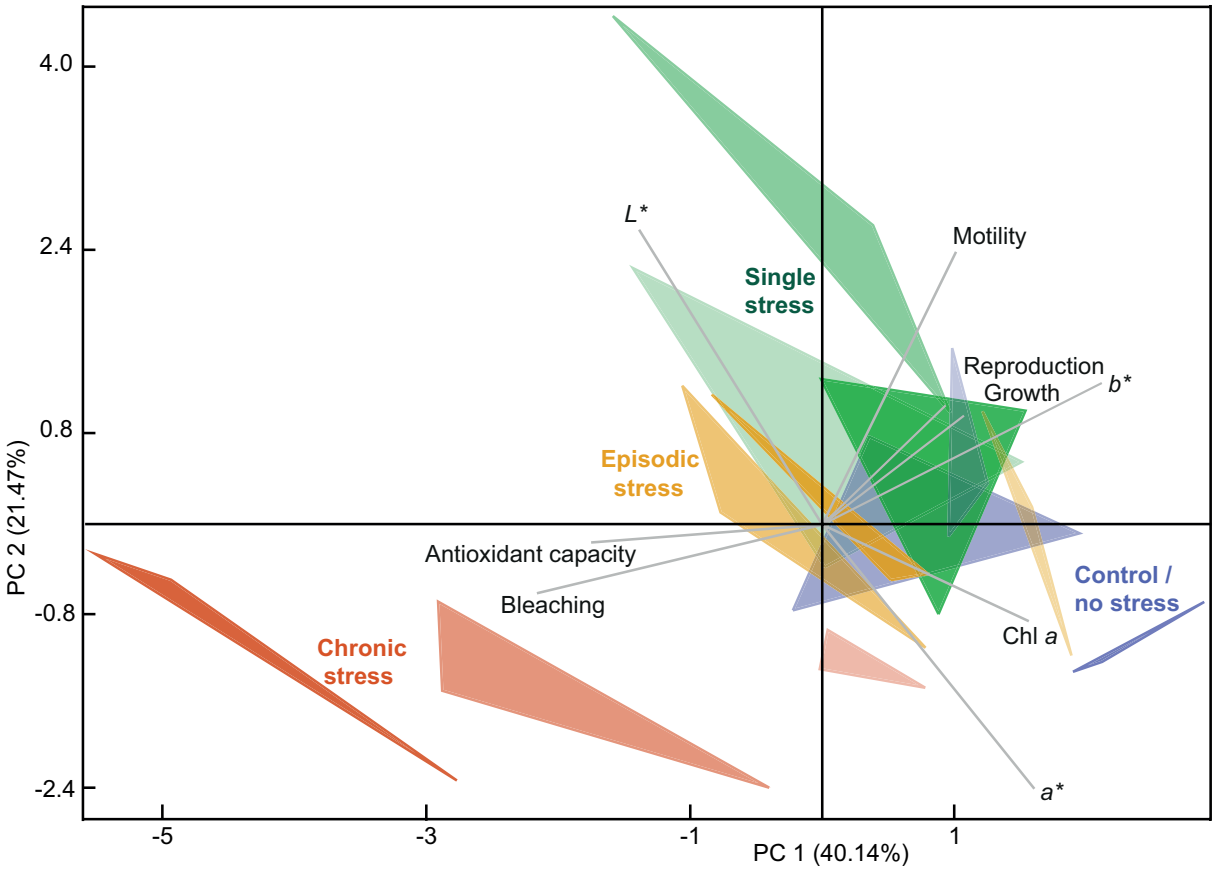

Figure 3-5. Principal component analysis (PCA) biplot visualizing experimental stress response patterns. The corners of the triangles represent the three replicates per thermal treatment: control / no stress (blue), single stress event (green), episodic stress events (yellow) and chronic stress (red); and the color intensity represents the temporal variable from day 12 (transparent), to day 21 and the final sampling time at day 30 (solid). The influence of partial bleaching frequency, CIE L*a*b* *color space values (*L* = whiteness, a* = green to magenta, b* = blue to yellow), Chl *a concentration, antioxidant capacity against peroxyl radicals (ACAP), growth rate, motility and mortality seen as empty shells (most likely indicating reproduction) are directionally indicated.*

3.4.1 Control treatment

Since the photosymbionts in the control treatment flourished, the host grew, reproduction likely occurred (seen as mortality), and ACAP values did not rise above natural population averages, we can use the experimental conditions and observed response patterns to predict how field populations respond. For instance, Chl *a* concentration increased over time, indicated by lower *L** and increasing *b** values, as well as greater net oxygen-production rates by the end of the experiment (Fig. S3-2). This response can be explained by natural increase in numbers of photosymbionts during the ontogeny of the foraminifera and may also reflect an increase symbiont density in response to low light levels in culture conditions (Talge and Hallock 2003; Williams and Hallock 2004) or a possible feeding-related rise in the availability of fixed nitrogen, which could increase the amount of nutrients supplied from the host to the symbionts. The former process of photo-acclimation is known from corals, which can increase the amount of chloroplasts in their

photosymbionts to meet their energy demands despite low-light conditions in their environment (Fabricius 2005) and might act similarly in LBF by increasing the amount of symbionts or their chloroplasts.

The ACAP values of the control population are comparable to values reported for a population of *A. lobifera* in the Great Barrier Reef, which shows elevated resilience towards temperature and nutrient stress, probably due to preconditioning based on environmental fluctuations (Prazeres et al. 2016b). Specifically, both the absolute ACAP values and the temporal trend in the control resemble those measured by Prazeres et al. (Prazeres et al. 2016b), indicating that the population of *A. gibbosa* in our study is possibly acclimatized or adapted to comparable conditions.

3.4.2 Chronic thermal stress

Chronic thermal stress induced gradual bleaching, which is reflected by reduced photopigment concentrations and ultimately decreasing photophysiological performance. This observation is in line with previous studies on LBF (Talge and Hallock 2003; Schmidt et al. 2011; Sinutok et al. 2011; Uthicke et al. 2011; Prazeres et al. 2016b). Although oxygen-production rates were negative after 30 days of chronic stress exposure, holobiont respiration rates indicated that the remaining photosymbionts were still photosynthetically active (Fig. S3-2). Those specimens that exhibited intense bleaching showed accumulation of brown material at the periphery of the shell and close to the aperture (Fig. S3-1d) resulting from the deterioration of chloroplasts, typically followed by degradation or expulsion of the photosymbiont residues (Talge and Hallock 2003; Schmidt et al. 2011). Despite survival of some photosymbionts, their decreased concentration and activity likely impaired the fitness of the holobiont, by reduced translocation of metabolites causing lower growth rates (Reymond et al. 2011; Schmidt et al. 2011, 2014, 2016b; Uthicke et al. 2011), reduced motility (Schmidt et al. 2011) and probably also less reproductive activity (Prazeres et al. 2017b) (here seen as mortality). Although growth rates across all treatments gradually slowed this is most likely due to the same natural aging trends known for benthic foraminifera (Hallock 1981). It is remarkable that that the chronic-stress treatment seems to have reduced growth by ~50% in comparison to the other treatments after the first measurements in the treatment. This early reduction in growth indicates that the primary response to chronic thermal stress is likely due to the holobionts using their energy to maintain homeostasis. Respiration rates could indicate that bacteria, which were feeding on the remains of dead foraminifers were respiring very actively. Alternatively, the respiration rates could indicate that *A. gibbosa* specimens from the chronic-stress treatment were still alive at the end of the experiment, although they did not reproduce, ceased to move, and did not grow after 21 days of chronic exposure.

Together with previous studies, our results support the hypothesis that foraminiferal hosts are more resistant to thermal stress than their endosymbionts (Talge and Hallock 2003; Fujita et al. 2014). Cytological analyses revealed that prolonged

temperature stress under low light conditions (6–8 µmol photons m^{-2} s^{-1}) induced significant declines in photosymbiont densities and lipid bodies, while some host endoplasm remained intact (Talge and Hallock 2003). In our experiment, similar exposure temperature and duration (32°C for one month) led to bleaching but was sub-lethal to the host, which reconfirms that LBF can survive bleaching, however with the overall reallocation of metabolic activity.

The lack of mortality in the chronic-stress treatment in our experiment seems to be at odds with other long-term chronic exposure studies, which showed increased mortality at elevated temperatures (Uthicke et al. 2011; Schmidt et al. 2014; Prazeres et al. 2016b). This could be related to variations between LBF and photosymbiont species, or durations and intensities of stress exposure in the different studies. However, the functionality of the holobiont at the end of our experiment appears to have been so severely impaired that more profound effects will likely ensue if stress continues or other interacting pressures occur (Schmidt et al. 2014). Hallock et al. (Hallock et al. 1995) reported a variety of issues associated with bleaching in *A. gibbosa*, including reproductive failure, epibiont infestations and calcification anomalies.

Here, we show for the first time that ACAP in *A. gibbosa* is greatly enhanced by chronic thermal stress (Figs. 3-4e and 3-5). The only other study to measure ACAP in LBF in response to thermal stress showed that after 30 days at 29°C, the ACAP of *A. lobifera* had not increased significantly (Prazeres et al. 2016b). The lack of ACAP response from *A. lobifera* compared to *A. gibbosa* from our study may have resulted from the 3°C higher exposure temperature in our experiment, species-specific temperature tolerances, different local adaptations or symbiont communities. The function of elevated ACAP is associated with defense mechanisms against amplified oxygen radicals produced by photosynthesis under higher temperature, as seen amongst cnidarians (Lesser 2006). Although the density of symbionts, which are expected to produce radical oxygen species, decreased over time (e.g., Fig. 3-4a) the ACAP increased continuously. This implies that either the remaining but more and more damaged symbionts were still producing sufficient oxygen radicals for the host's defense system to require higher ACAP to compensate for these, or that the antioxidant capacity was responding to the oxidative stress with a time lag. Since we did not measure gene or protein expression (Doo et al. 2012b; Mayfield et al. 2012), but on the level of enzyme and non enzymatic low-molecular-weight scavenger (e.g., glutathione, ascorbic acid, uric acid, vitamin E and carotenoids) capacity (Lesser 2006; Amado et al. 2009), these might be produced more slowly and, more importantly, might remain functional over considerably longer time periods. Overall, our chronic stress scenario suggests a reallocation of host energy towards defense and repair

mechanisms, thereby reducing calcification, motility and reproductive activity but preventing mortality.

3.4.3 Single and episodic stress events

The *A. gibbosa* coped well with fluctuating temperatures simulated by single and episodic thermal stress events. Most photosymbiont and holobiont response variables did not change significantly over the term of the experiment. This seems contradictory to former studies that analyzed the responses of LBF to short-term thermal stress, which found lower Chl *a* concentrations, reduced photosynthetic efficiency (Schmidt et al. 2011; Uthicke et al. 2011), and lower quantities of RuBisCO (Doo et al. 2012b) after hours to days of exposure. These studies, however, focused on the immediate response to stress, while our results represent their physiological response after they were released from the thermal stress. It is therefore possible that *A. gibbosa* and most of the photosymbiont variables (e.g., Chl *a* and color values) had already recovered within 24 hours after the peak thermal stress, demonstrating the capability of this species to quickly recover from short-term stress. Similarly, oxygen-consumption rates required only a few hours to recover from extreme temperatures, in contrast to photosynthesis rates that needed several days to recover (Fujita et al. 2014).

After the single stress event, net photosynthesis varied strongly over time. Because these variations were ongoing throughout the experiment, we interpret them as most likely related to the presumably high incidence of reproduction in this treatment (data lacking). Reproductive activity even exceeded the control specimens and represented the only variable in which single stress and episodic stress responses differed. Because half of the shells in the single-stress treatment were empty by the end of the experiment, the single thermal peak followed by stable conditions might have stimulated reproduction. In contrast, episodic stress appeared to suppress or delay reproduction in the same way as in the chronic-stress treatment. Correspondingly, suppression of asexual reproduction in adults and failure to normally calcify were reported from *A. gibbosa* specimens collected during summer, which also exhibited photosymbiont deterioration (Hallock et al. 1995). Previous studies (Hallock 1981; Prazeres et al. 2017b) related reduced reproduction and fecundity to low light intensities. Since in our study the light level was the same in all aquaria and reproduction presumably occurred in other treatments, this does not seem to be the driving factor here. In the case that recurring stress induces malfunction or impairment of reproductive activities, this would imply important long-term consequences for foraminiferal life cycles, population densities and community structures with severe impacts on carbonate budgets and overall health of coral-reef environments (Hallock et al. 1995; Doo et al. 2012a; Reymond et al. 2013; Prazeres et al. 2017b) and should be addressed in future studies.

3.4.4 Analogy to other coral reef calcifiers

While there are no comparable studies on the effects of fluctuating temperatures on LBF, other photosymbiotic reef organisms have been subjected to temperature variations and showed that even short temperature reductions can reduce immediate thermal damage within coral reefs. Such examples include large-amplitude internal waves, which cause pH and temperature to drop within minutes, allowing short-term relief, and have been shown to reduce the physiological effect of heat stress on corals (Buerger et al. 2015). Daily temperature fluctuations can be beneficial to the photosynthetic efficiency of coral larvae (Putnam et al. 2010), but led to strong declines in photosymbiont densities, while maintaining or even increasing calcification in studies on adult coral colonies (Putnam and Edmunds 2011). Corals that are exposed to extreme natural temperature fluctuations during spring-tide upwelling events increase most physiological and molecular parameters, suggesting that the holobiont may acclimate to fluctuating temperatures by the symbionts capacity to increase photosynthesis and carbon fixation (Mayfield et al. 2012). These results and our study support the hypothesis that temperature fluctuations, in contrast to chronic thermal stress, have substantially different effects on photosymbiotic reef calcifiers. The impact of thermal stress appears to not only depend on exposure level and duration, but also on whether the stress is constant or discontinuous because intermittent stress provides respite periods permitting repair mechanisms to alleviate or entirely prevent the detrimental effects of thermal stress. Interactive effects of multiple contemporaneous or consecutive stressors could produce different outcomes and should be targeted by future research.

Besides the immediate effects of temperature variations, thermal history is an important factor among photosymbiotic reef organisms, because local acclimatization or adaptation to thermal stress may enhance thermal resistance through higher phenotypic and metabolic plasticity. This is evident by elevated thermal tolerance in corals from habitats where they naturally experience temperature fluctuations, such as large-amplitude internal waves (Buerger et al. 2015) or lagoon pools (Oliver and Palumbi 2011b). Furthermore, coral colonies that were experimentally pre-stressed before exposure to severe prolonged thermal stress revealed more effective photoprotective mechanisms (Middlebrook et al. 2008). Similar to coral studies, *A. lobifera* populations from stable offshore environments are more sensitive to stress than those from inshore habitats that experience stronger fluctuating conditions (Prazeres et al. 2016b). Comparably, our results indicate that local conditions increased the tolerance of *A. gibbosa* to environmental changes, considering long-term subsurface temperature variability in the Florida Keys (Leichter et al. 2006). Specifically at the sampling location, Tennessee Reef situated in the Middle Keys, reefs were historically exposed to intermediate levels of sea-surface temperature variability (Soto et al. 2011). These intermediate thermal fluctuations seem to be beneficial to biodiversity, survival, and

recovery of the local stony-coral assemblages (Vega-Rodriguez et al. 2015). It is therefore highly probable that the population of *A. gibbosa* sampled for our experiment is adapted or acclimatized to thermal variability such that single- and episodic-stress treatments did not exceed its tolerance range. Indeed, time-series studies of *A. gibbosa* populations from the Florida Keys through the 1990s revealed that bleaching followed the solar cycle of irradiance, such that peak bleaching consistently occurred well before the late summer temperature maximum and the populations were typically already showing recovery when temperature peaked (Hallock et al. 1993, 1995).

No acclimatization to repeated stress events occurred in our study, but the LBF under chronic stress arrived close to the thermal tipping point. In some corals, elevated thermal tolerance can be independent of local variation in ocean temperature, such that their acclimatization capacity to future warming is limited (Rodolfo-Metalpa et al. 2014). Whether *A. gibbosa* is generally characterized by high thermal tolerance or if the high physiological plasticity found in this study is specific to the local population assessed, which would suggest a high acclimatization capacity, has to be targeted in future research. This raises the discussion on whether the resilience of these foraminifers is a product of short-term acclimatization due to recent thermal history, or if long-term adaptation has increased the tolerance of these photosymbiotic calcifiers. Such questions could be disentangled with the use of 'omics' approaches, which can determine the influence of environmental stressors on the gene or protein level and therefore reveal meaningful insights into underlying molecular processes governing acclimatization/adaptation pathways. Furthermore, research on the flexibility and physiological plasticity of the photosymbiont community would further improve our understanding of LBF adaptive potential.

3.5 Conclusions

Our laboratory experiment represents the first study focusing on the physiological responses of LBF to temperature fluctuations. Although some physiological responses showed high variability, this study illustrates how thermal variation has different effects on the foraminifera and their photosymbionts compared to chronic exposure despite the same peak temperature. We also showed how reoccurring stress did not induce acclimatization, likely because *A. gibbosa* populations from the Florida Keys are already adapted to the applied pattern and amount of temperature variability. This study, together with coral research, conveys how temperature fluctuations affect reef ecosystems differently than chronic exposure, provided that the intensity and duration of transient thermal stress events do not exceed naturally occurring extremes (Soto et al. 2011; Vega-Rodriguez et al. 2015). This study further demonstrates that experimental studies and projections of global change effects on reef calcifiers must consider temporal fluctuations in stress

exposure. In a warming ocean, fluctuations in stress level can be an important factor to facilitate recovery from chronic heat stress (Buerger et al. 2015), which either allow for short-term acclimatization (Mayfield et al. 2012), or induce physiological acclimation (Middlebrook et al. 2008) by enhancing metabolic efficiency (Putnam et al. 2010). The energetic costs of acclimatization through high physiological plasticity (Boyd et al. 2016), such as possible suppression of reproduction, are important aspects that need to be addressed in future research. Overall, marine habitats with fluctuating temperature regimes may bear highly resilient reef calcifiers with a high potential to seed or serve as potential reef refugia (Leichter et al. 2006; Buerger et al. 2015; Vega-Rodriguez et al. 2015), and therefore need to be primary focal points of coral reef research to guide global conservation efforts.

3.6 Acknowledgments

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3.7 Supplementary materials

Table S3-1. Results of Tukey's HSD post hoc test for Treatment × Time interactions. All variables that showed significant interactions between Treatment × Time in the ANOVA (P-value < 0.05 in Table 3-2) were followed by Tukey's HSD test procedure to further identify individual differences between treatments (1 = control / no stress, 2 = single stress event, 3 = episodic stress events, 4 = chronic stress) at each sampling day (time). For respiration and net photosynthesis the ANOVA was performed (a) only for treatments 1, 2 and 4 on all sampling days or (b) only on sampling days 3, 12 and 21 for all treatments.

Variable	Time (day)	Treatment	Estimate	SE	t-ratio	*P*-value
*b**	3	1 - 3	7.063	1.643	4.30	0.001
		2 - 3	7.379	1.643	4.49	0.001
		3 - 4	-4.944	1.643	-3.01	0.025
	12	1 - 2	6.124	1.643	3.73	0.004
		1 - 4	9.757	1.643	5.94	< 0.001
		3 - 4	5.914	1.643	3.60	0.006
	21	1 - 3	6.044	1.643	3.68	0.005
		1 - 4	13.028	1.643	7.93	< 0.001
		2 - 4	11.019	1.643	6.71	< 0.001
		3 - 4	6.984	1.643	4.25	0.001
	30	1 - 3	5.406	1.643	3.29	0.012
		1 - 4	18.490	1.643	11.25	< 0.001
		2 - 4	14.520	1.643	8.84	< 0.001
		3 - 4	13.084	1.643	7.96	< 0.001
Chl *a*	21	1 - 2	0.208	0.060	3.45	0.010
		1 - 3	0.217	0.060	3.60	0.007
	30	1 - 2	0.223	0.060	3.70	0.005
		1 - 3	0.278	0.060	4.62	0.001
		1 - 4	0.457	0.060	7.59	0.000
		2 - 4	0.235	0.060	3.90	0.003
		3 - 4	0.179	0.060	2.98	0.030
Net photosynthesis[a]	12	1 - 2	2.508	0.701	3.58	0.006
		2 - 4	-2.393	0.701	-3.41	0.009
	21	2 - 4	2.321	0.701	3.31	0.011
	30	1 - 4	2.661	0.701	3.79	0.004
		2 - 4	2.173	0.701	3.10	0.017
Net photosynthesis[b]	12	1 - 2	2.508	0.771	3.25	0.017
		2 - 4	-2.393	0.771	-3.11	0.024
	21	2 - 4	2.321	0.771	3.01	0.029
Respiration[a]	12	1 - 2	1.131	0.364	3.11	0.013
Respiration[b]	3	3 - 4	1.025	0.358	2.87	0.040
	12	1 - 2	1.131	0.358	3.16	0.021
	21	1 - 2	0.208	0.060	3.45	0.010
ACAP	12	1 - 3	0.645	0.226	2.85	0.039

	30	1 - 4	-1.147	0.226	-5.08	0.000
		2 - 4	-0.818	0.226	-3.62	0.006
		3 - 4	-0.838	0.226	-3.71	0.005
Motility	21	1 - 2	-1.433	0.311	-4.61	< 0.001
		2 - 3	1.133	0.311	3.64	0.005
		2 - 4	1.700	0.311	5.47	< 0.001
	30	2 - 4	1.333	0.311	4.29	0.001
		3 - 4	1.383	0.311	4.45	0.001

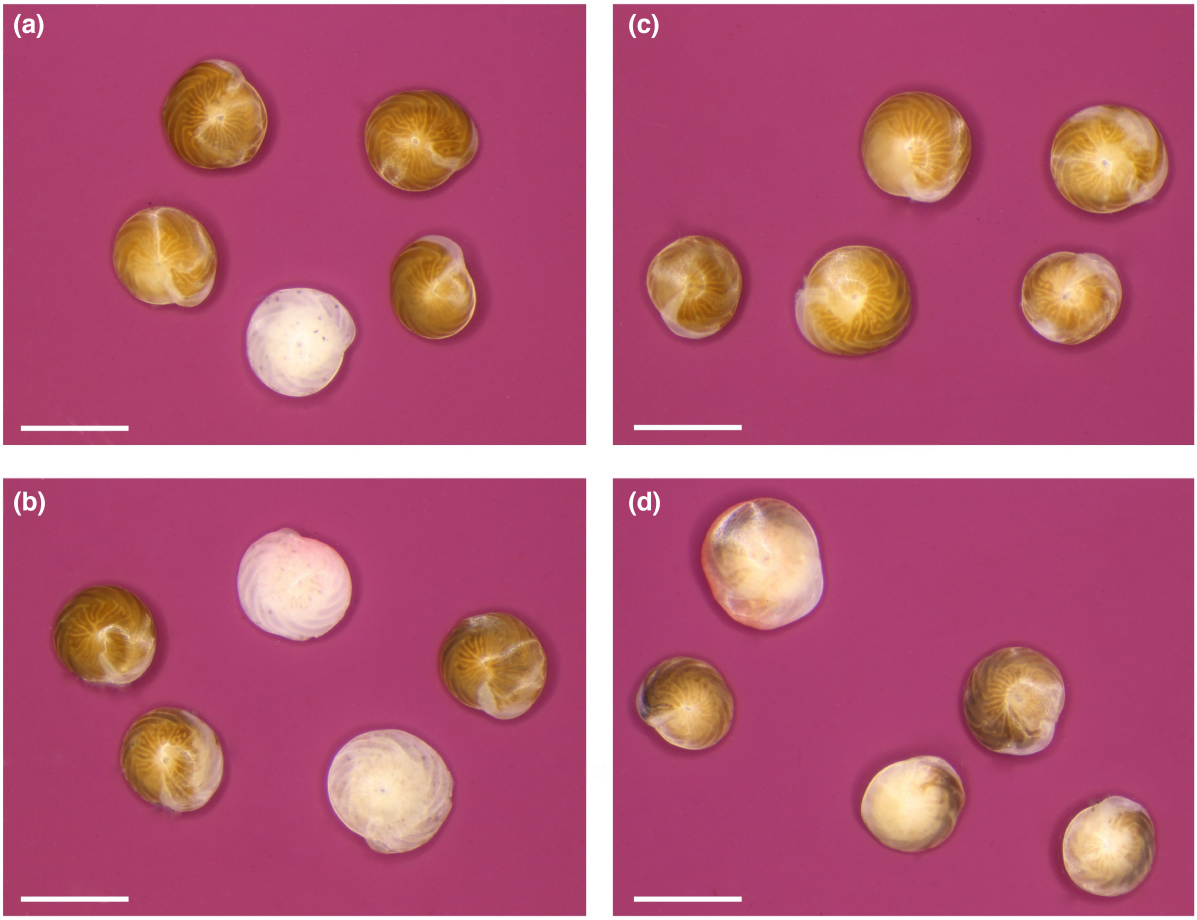

Figure S3-1 Representative photos of Amphistegina gibbosa *after exposure to different thermal-stress treatments for 30 days. The images illustrate changes in holobiont color and appearance of empty shells, representing one of the three replicates in each of the treatments: (a) control (no stress), (b) single stress event, (c) episodic stress and (d) chronic stress. Individuals in (a) and (b) that turned entirely white (empty tests) died or underwent reproduction, some specimens in (b) and (c) showed mottling / partial bleaching and severely impacted foraminifera in (d) bleached strongly, but at the same time showed accumulation of dark materials at the shell periphery. White scale bars represent 1 mm length.*

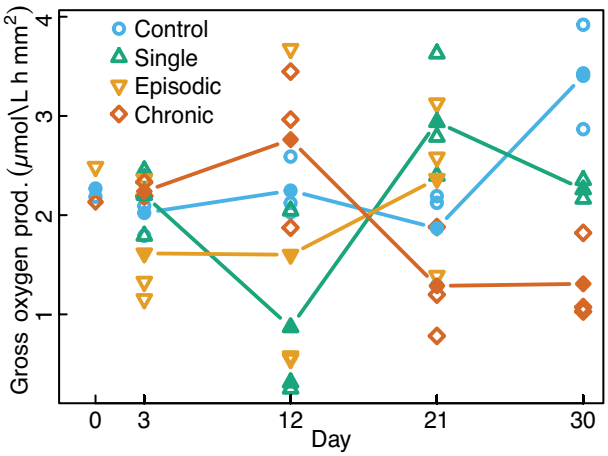

Figure S3-2. Gross photosynthesis rates of A. gibbosa *in response to different thermal-stress treatments. The gross photosynthesis is expressed as oxygen production per surface area at time zero and subsequently after each episodic thermal-stress event in the treatments: control / no stress (blue circles), single stress event (green triangles), episodic stress events (yellow inverse triangles) and chronic stress (red diamonds). Filled symbols connected by lines represent the running means of each treatment (n=3) on the respective sampling time (day).*

Chapter Four

DISENTANGLING THERMAL STRESS RESPONSES IN REEF-CALCIFIERS AND THEIR PHOTOSYMBIONTS BY SHOTGUN PROTEOMICS

This work is under review at *Scientific Reports*

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Personal contribution to chapter 4:

Idea and concept:
M. Stuhr developed the idea of using proteome analysis to differentiate between host and symbiont stress responses, with contributions on methodology from B. Blank-Landeshammer, L. Kollipara and A. Sickmann.

Research:
M. Stuhr carried out the experiment. B. Blank-Landeshammer and L. Kollipara adapted the proteomics workflow for foraminifera samples and conducted proteome analysis, with contributions from M. Stuhr.
M. Stuhr and B. Blank-Landeshammer assembled the reference database, analyzed the data and created plots and tables.
M. Stuhr interpreted the data.

Writing and figure preparation:
M. Stuhr wrote the initial manuscript and prepared the figures, with contributions on method descriptions from B. Blank-Landeshammer and L. Kollipara.
C.E. Reymond, H. Westphal, M. Kucera L. Kollipara and B. Blank-Landeshammer made improvements.

Abstract

The proliferation of key marine ecological engineers and carbonate producers often relies on their association with photosymbiotic algae. Evaluating stress responses of these organisms is important to predict their fate under future climate projections. Physiological approaches are limited in their ability to resolve the involved molecular mechanisms and attribute stress effects to the host or symbiont, while probing and partitioning of proteins cannot be applied in organisms where the host and symbiont are small and cannot be physically separated. Here we apply a label-free quantitative proteomics approach to detect changes of proteome composition in diatom-bearing benthic foraminifera experimentally exposed to three thermal-stress scenarios. We developed a workflow for protein extraction from less than ten specimens and simultaneously analyzed host and symbiont proteomes. Despite little genomic data for the host, 1,618 proteins could be partially assembled and assigned. The proteomes revealed identical pattern of stress response among stress scenarios as that indicated by physiological measurements, but allowed identification of compartment-specific stress reactions. In the symbiont, stress-response and proteolysis-related proteins were up-regulated while photosynthesis-related proteins declined. In contrast, host homeostasis was maintained through chaperone up-regulation associated with elevated proteosynthesis and proteolysis, and the host metabolism shifted to heterotrophy.

4.1 Introduction

Most marine reef-building organisms such as corals rely on symbiosis with photosynthesizing microalgae. Tropical coral reef ecosystems are one of the structurally most complex and richest hotspots of biodiversity on Earth (Odum and Odum 1955). The symbiotic relationship provides the holobiont with a clear advantage in nutrient–limited settings, but it comes at a cost of lower resilience to perturbations (Stanley Jr and Lipps 2011; Raharinirina et al. 2017). Indeed, marine photosymbiotic ecosystems are threatened and decline rapidly due to human activity. Global warming is currently considered as the most damaging factor (Hughes et al. 2017), inducing bleaching responses in reef organisms, i.e., the loss of photosynthetic microalgae and/or photo-pigments (Hoegh-Guldberg 1999; Lesser 2006). Massive bleaching events are reported more frequently, already becoming a regular occurrence (Baker et al. 2008; Hughes et al. 2017). Ocean warming rarely affects marine ecosystems directly by elevated mean seawater temperature. Instead, heat stress is often induced during episodic heating events. Subsequently, research on thermal tolerance of marine photosymbiotic organisms has shifted from determining thermal limits under constant exposure to the consideration of different thermal stress frequency scenarios. Studies on photosymbiotic corals and large benthic foraminifera (LBF), i.e., photosymbiont-bearing calcifying eukaryotes, have demonstrated that, compared to chronic stress, transient heat-stress events have little immediate impact on these organisms

(Mayfield et al. 2012; Wall et al. 2015; Stuhr et al. 2017). On the contrary, variable temperature regimes might even increase their resilience towards warming, because fluctuations may facilitate acclimatization and promote recovery from heat stress events (Mayfield et al. 2012; Boyd et al. 2016). The possible energetic costs and underlying molecular mechanisms of this acclimatization (Boyd et al. 2016) are important aspects that need to be understood in order to make projections on adaptive capacity and resilience potential of coral reef organisms.

The most direct approach to reveal the molecular mechanisms of resistance and adaptation to thermal stress involves the analysis of proteome composition. In symbiont-bearing organisms the stress response involves two compartments (host and symbiont) and appropriate methods are needed to disentangle the specific proteome responses (Mayfield et al. 2016b). Due to high sensitivity and high throughput at relatively low costs, 'omics' approaches have advanced quickly, providing promising new insights in the molecular mechanisms of stress responses in symbiont-bearing marine organisms (Oakley et al. 2016; Farag et al. 2016; Mayfield et al. 2016a). Whilst proteomics provides powerful tools to understand how stress affects the biology of organisms, there are limitations to its application. Mass spectrometry based proteomics is strongly dependent on the coverage of sequence databases, which can be especially challenging in environmental research and limits its applications to well-studied model organisms. This bottleneck can be circumvented by performing cross-species homology searching by hybrid *de novo* peptide sequencing and database search approaches (Yonghua Han et al. 2005; Ma and Johnson 2012). Furthermore, previous studies have focused on non-symbiotic diatoms (Luo et al. 2014; Boyd et al. 2015; Muhseen et al. 2015) or only the host proteomes (Oakley et al. 2016, 2017), while invertebrate and algae proteomes are rarely analyzed simultaneously. This is possible by performing proteomics analysis of holobionts and annotating the peptides/proteins *in silico* to either host or symbionts (Weston et al. 2015; Mayfield et al. 2016a). By targeting photosymbiotic organisms as a functional unit, including the host and the endosymbiotic algae, key interactions between both compartments can be detected.

The ideal organism to test this approach and reveal compartment specific molecular response to thermal stress are symbiont-bearing benthic foraminifera, such as the circum-global genus *Amphistegina*, which inhabits oligotrophic coral reef environments, hosts diatom photosymbionts (Langer and Hottinger 2000) and is a vital constituent of coral reef ecosystems (Hallock 2005). Due to their physiological sensitivity (Zmiri et al. 1974) they are commonly used as bioindicators for past and present coral reef health (Williams et al. 1997; Hallock et al. 2003; Cooper et al. 2009) and provide a useful model to study the effects of environmental change on photosymbiotic calcifiers. Their algal symbionts enable these calcium carbonate producers to generate approximately 3.9–5.4% of reef carbonate sediments (Doo

et al. 2016). Both chronic thermal stress and high light intensities can induce bleaching in LBF, which is usually accompanied by a multitude of other afflictions and can ultimately diminish populations and reduce carbonate accumulation (Talge and Hallock 2003; Hallock et al. 2006; Reymond et al. 2011; Schmidt et al. 2011; Fujita et al. 2014; Prazeres and Pandolfi 2016; Stuhr et al. 2017).

Long-term heat stress appears to affect LBF primarily by disturbing the photosynthetic performance of the symbionts (Talge and Hallock 2003; Reymond et al. 2011; Schmidt et al. 2011; Uthicke et al. 2011), causing reduced holobiont calcification and growth (Reymond et al. 2011; Uthicke et al. 2011; Prazeres and Pandolfi 2016; Stuhr et al. 2017) and reducing host activity (Schmidt et al. 2011; Stuhr et al. 2017). In contrast, they display a marked capacity for acclimatization to short-term thermal stress events that do not induce bleaching (Stuhr et al. 2017), but the exact mechanisms of acclimatization and thermal stress response remain unresolved. Existing protein expression studies revealed decreases in the rate-limiting carbon fixation enzyme ribulose 1-5-biphosphate carboxylase/-oxygenase (RuBisCO) (Doo et al. 2012b) and high ratios of the 70kDa stress protein (Heinz et al. 2012) in response to heat shocks. These gel-based approaches can only target specific proteins, are challenging to apply to small protein volumes and do not allow partitioning between host and symbionts (Doo et al. 2014b).

In order to reveal insights into their mechanisms of response to ocean warming, we carried out an experiment comparing three thermal stress scenarios (Stuhr et al. 2017). Here, *A. gibbosa* populations were exposed to (a) no thermal stress i.e., control conditions at constant 25.5°C, (b) a single thermal stress event of three days up to 32°C followed by control conditions, (c) episodic thermal stress events alternating with periods of six days at control conditions and (d) chronic thermal stress at 32°C over one month (Fig. S4-1). Alongside quantification of classical physiological response parameters, a subset of specimens from the same experiment has been used for a label-free proteome analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS), allowing simultaneous evaluation of the host and symbiont compartments. The experimental setup combining proteomics with physiological measurements allows us to (i) authenticate the dual-compartment approach on non-model organisms, (ii) elucidate response mechanisms induced by single and episodic thermal stress, and (iii) determine the underlying molecular response to chronic thermal stress, including LBF bleaching.

4.2 Results and interpretation

4.2.1 Dual-compartment protein identification

In this study, a total of 1,618 proteins belonging to the concatenated host-symbiont database were identified by homology-driven search approaches, all of which were present in samples of all treatments and at the beginning of the experiment. In order to condensate these protein sequences – stemming from closely related organisms – a similarity-based clustering step was performed, which condensed 1,618 protein sequences to 1,136 protein clusters (supplementary Table S4-3). The largest cluster comprised 10 protein sequences (all related to various actin isoforms), while 926 proteins remained as single-protein clusters. Out of all protein clusters, approximately 31% were assigned to the host foraminifera and 68% to the symbiont compartment. Nine clusters contained sequences stemming from both host and symbionts and could not be clearly associated to a compartment, likely because the respective protein sequences are highly conserved. Protein clusters will be simply referred to as proteins in the following sections.

4.2.2 Multivariate analysis of relative protein abundance changes

The level of change in protein abundances infers different effects of elevated water temperatures on *Amphistegina gibbosa*. Significant variations between treatments were observed, particularly under chronic thermal stress (Fig. 4-1a). These directional proteome developments in different treatments were characterized by the trend and amount of change in abundance of regulated proteins (p-ANOVA $\leq$ 0.05). The proteome responses are largely in agreement with the physiological variables investigated within the same experiment (Stuhr et al. 2017) and therefore demonstrate high congruency of the outcomes of both approaches (Fig. 4-1b). This is understood as verification of the credibility of our dual-compartment proteomics approach. Additionally, future availability of transcriptome or whole genome sequencing data could further boost the sensitivity of our proteomic analysis and be used to re-analyze the obtained data to gain further insights not attainable by homology-based search methods.

More than 25% of all identified proteins were regulated with respect to the control (Figs. 4-2a, S4-2 and S4-3). The small extent of regulation between the single-stress treatment and only slightly altered development in response to episodic stress, in contrast to the discrete impact of chronic-stress treatment, reflects the LBF holobionts' capability to maintain or quickly restore biological functions/homeostasis during or after thermal stress events. In contrast to the stress-event treatments, LBF exposed to chronic stress underwent distinct changes, indicating that the nature of the proteome response to thermal stress is depending on the persistence of stress exposure. The analysis illustrates that those proteins assigned to the host compartment are mostly distributed in direction of the chronic-stress treatment (Fig. S4-3). The foraminifers' reaction hence plays a more

prominent role in continuous stress exposure, while proteomic changes in response to stress-event treatments took place dominantly in the symbiont compartment.

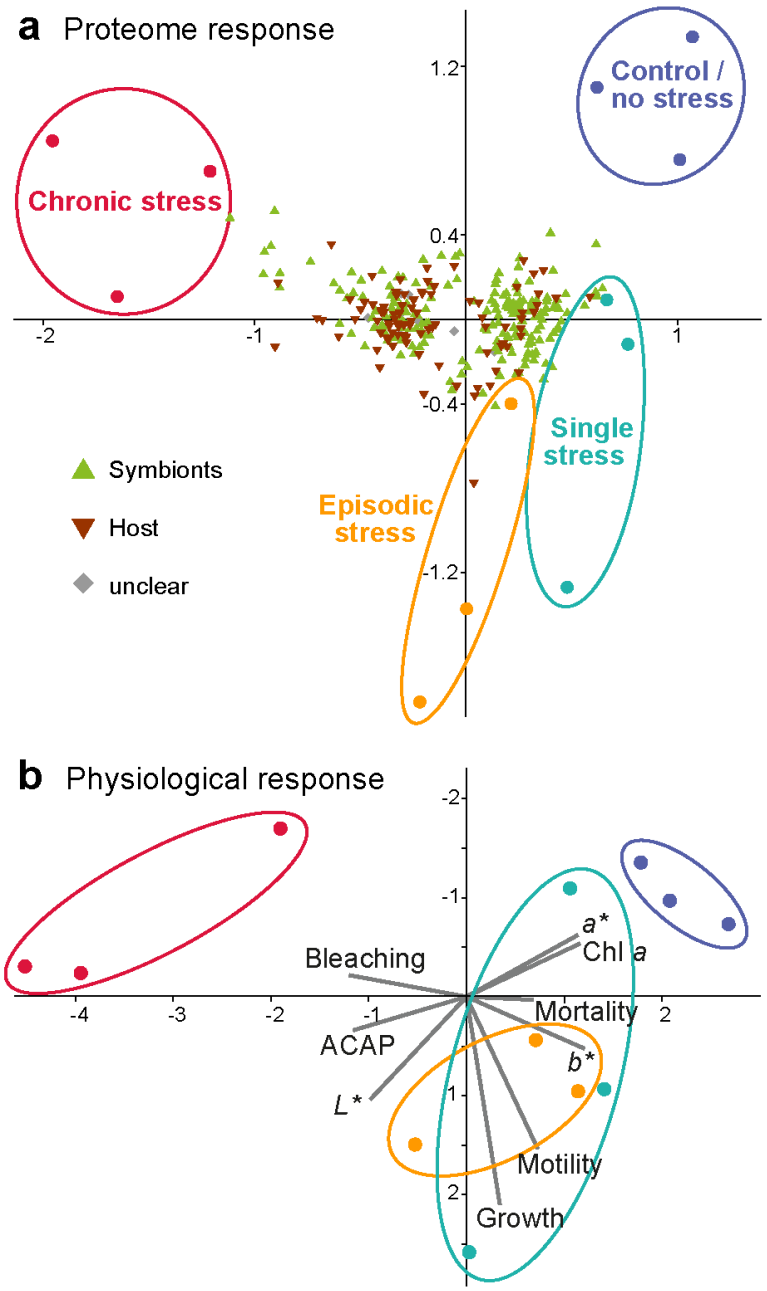

Figure 4-1. Comparison of proteomics results with the previously published physiological response of Amphistegina gibbosa *in response to a single short-term stress event (turquoise), episodic stress events (orange) or chronic thermal stress (red) compared to the control treatment (blue). (a) Correspondence analysis of relative protein abundances of all 294 regulated proteins, showing the distribution of proteins (host = brown inverse triangles, symbiont = green triangles, unclear/both = grey diamonds) that drive the directional changes between treatments. 65.8% are explained by variation along axis 1 and 11.2% by axis 2. (b) Principal component analysis of physiological metrics of LBF from the same experiment (Stuhr et al. 2017), including chlorophyll a concentration (Chl a), total capacity against peroxyl radicals (ACAP), motility, growth rate, CIE L*a*b* color space values (L* = whiteness, a* = green to magenta, b* = blue to yellow), mortality and bleaching frequency. 60.1% are explained by variation along principal component 1 and 18.6% by principal component 2.*

With respect to the start condition (Fig. S4-7), the LBF proteomes in all treatments evidently underwent changes, likely resulting from ontogenetic development or an on-going acclimation of the LBF to culture conditions. Since these proteomic alterations influenced all treatments equally and are outside the scope of this study, they will not be further discussed here. For the sake of complete documentation, data and figures with respect to start conditions are available in the supplementary material (Table S4-3 and Figs. S4-8 – S4-10).

4.2.3 Proteomic responses to thermal stress

To gain more precise insights into the most important cellular mechanisms occurring in response to the different thermal-stress scenarios, we here now focus on the differently abundant proteins (DAPs), i.e., those regulated proteins that could be clearly assigned to one of both compartments, showed significant variations between treatments in Tukeys' HSD post hoc test (p-value ≤ 0.05) and a distinct positive or negative change in abundance ($\log_2$ FC > 1 or < –1). With these stringent criteria, 49 proteins in the host (Fig. 4-2b) and 108 proteins of the symbiont compartment (Fig. 4-2c) were differentially abundant in comparison to the control. They are summarized in the supplementary Tables S4-1 and S4-2, where they are organized by $\log_2$ fold changes and color-coded by general biological functions and processes. These are not exclusive but rather describe their most prominent general cellular roles. The complete list of GO term annotations, including assignment of molecular functions, biological processes and cellular components, is given in the supplementary Table S4-4.

The single stress-event treatment only induced four DAPs in total. The three-day stress peak at the beginning of the experiment thus had very little influence on the holobiont proteome in the long-term. Both compartments showed more pronounced responses in reaction to episodic stress events. In the symbiont compartment, the increased DAPs belonged to protein folding and degrading categories that are typically increased in response to stress in order to maintain homeostasis. Depleted DAPs incorporated some proteins participating in photosynthesis and other carbohydrate metabolizing processes, indicating a slight reduction of carbon concentrating mechanisms. Overall, a minor stress response was detected, but no lethal impacts on the diatoms derived from the episodic stress events. Interestingly, some processes in the host seemed to be slightly stimulated by the thermal fluctuations. Although actin, serine peptidase, and serine palmitoyltransferase were depleted, suggesting that the cytoplasm might have been damaged, the actin-related protein 2 was increased along with few metabolic and biosynthesis-related proteins. Since this protein mediates actin nucleation, new cytoskeletal filaments were likely created and/or actin-cytoskeleton based processes like cell locomotion, phagocytosis or intracellular motility of vesicles were enhanced (Travis and Bowser 1986). These results are at odds with the proteomic responses of corals that show down-regulation of Hsps as well as proteins involved in translation and metabolic processes when exposed to quarter-daily temperature fluctuations (Mayfield et al. 2016a). Yet our previous observations reflect the proteomic outcome as holobiont motility and growth rates were also highest in this treatment (Stuhr et al. 2017). Whether the foraminifers activity was enhanced as a general stress reaction, for repair or acclimatization, or if thermal variations act positively on the holobionts performance could not be determined here. However,

a fast reaction to the recently experienced thermal fluctuations could be crucial for their resilience to environmental variations.

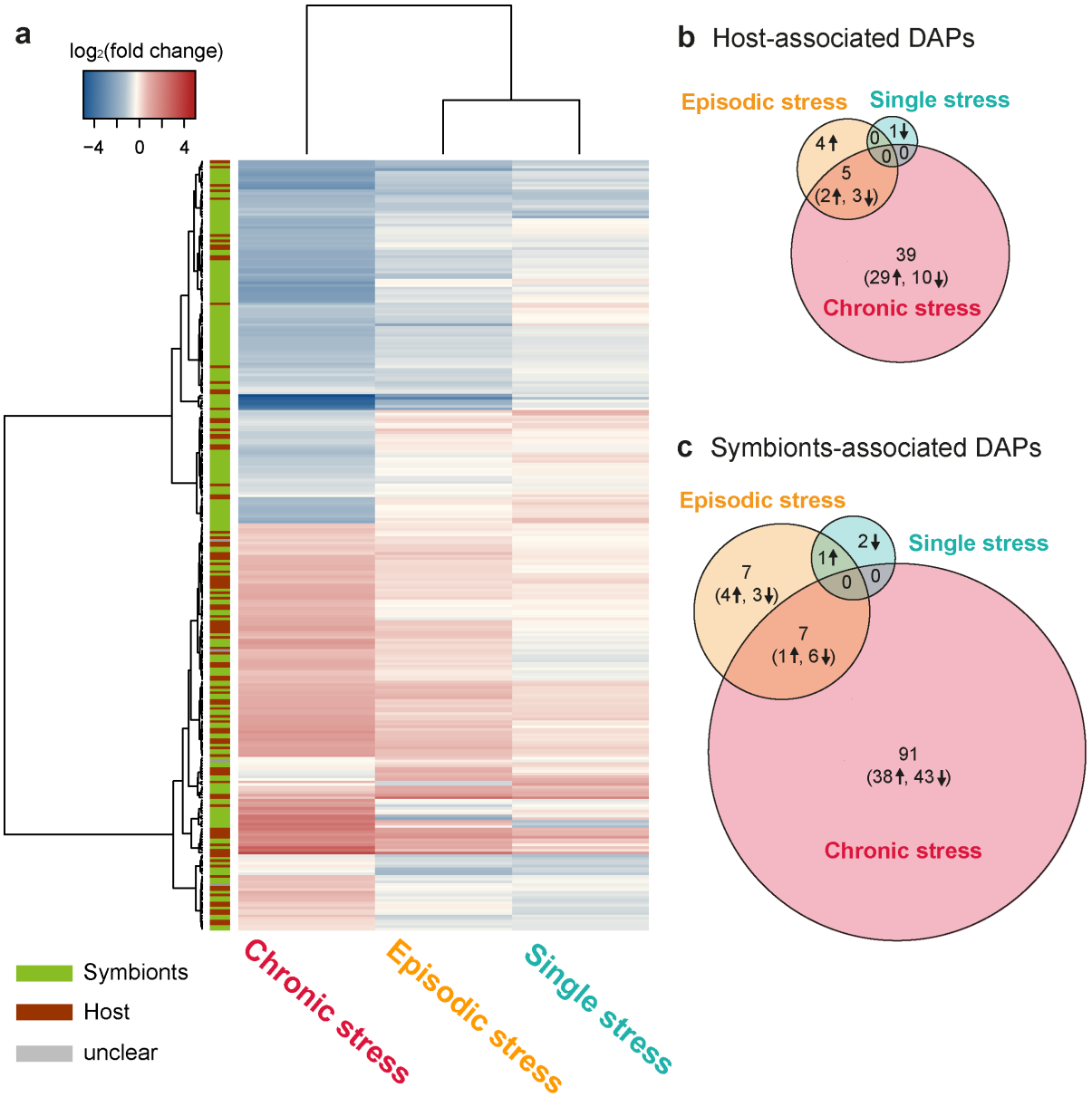

Figure 4-2. The heatmap with hierarchical cluster analysis (Euclidean distance) of all regulated proteins compared to the control (a) illustrates the direction of log_2 fold changes in protein abundances and their distribution among host foraminifera (brown) and symbionts (green) in Amphistegina gibbosa. *The normalized abundance values, significant p-values, and accessions of all regulated protein clusters are reported in supplementary Table S4-3. Venn diagrams show the amount of proteins in* A. gibbosa *that significantly changed in abundance (DAPs) in response to a single stress event (turquoise), episodic stress events (orange) and chronic stress (red) compared to the control in (b) the host (n = 49) and (c) the symbiont compartment (n = 108). Overlapping areas show protein groups that were equally regulated in more than one treatment. Arrows indicate how many proteins were up (↑) or down (↓) regulated.*

4.2.4 Compartment-specific impacts of chronic thermal stress

The most and strongest changes in protein abundances of both compartments were induced by chronic thermal stress, impacting a multitude of functions and processes (Figs. 4-3 and S4-4 – S4-6). Together, these suggest that the following cellular responses and changes in pathways occurred in the chronic-stress treatment:

Symbionts – Oxidative stress, protein folding and degradation

Heat stress in algae usually leads to the production of reactive oxygen species (ROS) by a malfunctioning of the photosystems, generating oxidative stress that may severely damage the cells(Lesser 2006). Our detection of significantly elevated DAPs that are usually responsible for protein quality control, folding and degradation (e.g., Hsps, calreticulin, prohibitin) affirm that protein and cell damage occurred. Predominantly, members of the Hsp70 family were elevated, corresponding to the temperature-induced increase in Hsp gene expression in diatoms described previously (Leung et al. 2017). The production of these chaperones, which directly mediate correct folding and bind to partially denatured proteins to prevent their aggregation, is usually stimulated by proteotoxic stress, but also the synthesis of new proteins requires chaperones for assistance in folding (Gethering and Sambrook 1992). Here, we also detected a remarkable up-regulation of proteins responsible for cell death and degradation such as the hypersensitive-induced response (Mur et al. 2008) and an autophagy-related protein (Atg8) (Nakatogawa et al. 2009), indicating programmed cell death. Hypersensitive response is a mechanism that in plants is usually observed as a final stage in reaction to pathogens that leads to the rapid death of infected cells, possibly triggered by the presence of ROS (Mur et al. 2008). Atg8 is an ubiquitin-like protein that is essential for the formation of autophagosomes, which sequester bulk cell materials to be degraded and deliver them to the vacuoles, but it is also thought to play a key role in selective autophagy (Nakatogawa et al. 2009). Moreover, calreticulin/calnexin, which binds to defective or incorrectly folded proteins and thereby targets them for degradation, and at least one member of the Clp proteases family (Page and Di Cera 2008) that often act in selective proteolysis (Gottesman et al. 1990) were elevated. Similarly, the impairment of the photosynthetic electron transport and respiratory chains in iron deprived diatoms results in excess ROS accumulation, likely inducing redox-sensitive mechanisms that regulate metabolic rates in order to ensure cellular homeostasis (Muhseen et al. 2015) or triggering programmed cell death (Luo et al. 2014).

In contrast to an expected up-regulation of antioxidants, the only recognized peroxidase (phospholipid methyltransferase) detected in the symbiont compartment was strongly depleted. This suggests that the amount of oxygen radicals might have surpassed the available ROS scavengers and could thus have damaged lipids, proteins and DNA (Lesser 2006). Interestingly, we found a severe

reduction of HopJ type III effector proteins (hypersensitive response and pathogenicity-dependent outer protein), which usually play key roles in the host-pathogen interactions in the type III secretion system of pathogenic bacteria (Collmer et al. 2000). These pathogens cause diverse diseases in hosts, based on their ability to colonize the intercellular spaces of plant tissues and cause death. As effector proteins usually help pathogen to invade host tissue and suppress its immune system, analogous functioning proteins might be crucial to keep the symbiosis in LBF intact, i.e., to protect the diatoms inside the host cell from being destructed. Considering possible interactions that might cause the observed degradation of endosymbionts during bleaching in LBF, such mediators should be studied in more depth. Overall, the strong expression of chaperones and degradation-related proteins shows that exposure to heat did not only induce repair mechanisms, but also cell death and protein degradation in the photosymbionts. As we observed severe bleaching of our heat stress specimens (Stuhr et al. 2017), this is in line with prior studies (Schmidt et al. 2011; Muhseen et al. 2015; Stuhr et al. 2017) and shows that prolonged thermal stress considerably harms the performance and induces deterioration of diatom symbiont cells (Fig. 4-4a).

Symbionts – Reduction of carbon fixation and core metabolism

Because of their evolutionary history, diatom metabolism pertains some exceptional features in comparison to other photosynthetic eukaryotes (e.g., green algae and land plants), such as the presence of enzymes more commonly found in prokaryotes and the possession of the enzymatic machinery required for C4 carbon fixation (Singh et al. 2015). Additionally, the mode of CO_2 concentration and the post-translational regulation of photosynthetic products are highly complex and remain largely unknown among diatoms (Kroth et al. 2008). In our study, the symbiont-associated DAPs that decreased strongest comprised a variety of chloroplastic proteins and enzymes involved in photosynthesis and the coupled carbon-fixating metabolism (e.g., RuBisCO, fucoxanthin chlorophyll *a/c*). Carbohydrate metabolic processes, specifically the production of polysaccharides via photosynthesis and the Calvin cycle, seemed to be severely impaired by the damaging effects of chronic heat exposure. Consequently, also DAPs participating in conversion of the resulting carbohydrates into storage compounds (Singh et al. 2015) or their utilization via glycolysis or parallel metabolic pathways (Kroth et al. 2008) appeared to be diminished. These pathways represent major sources of cellular energy (mostly stored and transported in the form of ATP) and provide the precursors that are necessary for the synthesis of many essential biomolecules such as lipids. The deficiency of CO_2 concentrating mechanisms was hence similarly detectable by the severe reduction of chloroplastic ATPases and other proton transporters.

Although proteomic responses of diatoms to several environmental stressors have been studied (Luo et al. 2014; Muhseen et al. 2015) and temperature was identified as the major driver influencing diatom growth (Boyd et al. 2015), no comparable studies have focused on endosymbiotic living diatoms. In the planktonic diatom *Pseudonitzschia multiseries*, elevated temperature leads to enhanced intracellular protein transport and turnover (Boyd et al. 2015). Other studies determined optimal temperature for the model diatom *Thalassiosira pseudonana* to be around 25°C, with growth and photosynthetic performance clearly dropping at higher temperatures (Leung et al. 2017). Likewise, high water temperatures have been formerly shown to lead to decreased photosynthetic efficiency in other LBF (Schmidt et al. 2011; Uthicke et al. 2011) and might even induce photoinhibition (Fujita et al. 2014). Heat shock experiments on the dinoflagellate-bearing LBF *Baculogypsina sphaerulata* demonstrated significant decreases in the expression of RuBisCO at 34°C, but not at temperatures up to 32°C (Doo et al. 2012b). As days to weeks of exposure to sub-lethal heat can severely decrease photosynthetic efficiency (Schmidt et al. 2011; Uthicke et al. 2011), our results support the hypothesis that the heat-induced reduction in RuBisCO may diminish carbon fixation (Doo et al. 2012b) seen through reduced calcification and holobiont growth (Reymond et al. 2011; Schmidt et al. 2011; Prazeres and Pandolfi 2016; Stuhr et al. 2017). The observed depletion of photopigment concentrations and clearly decreasing but still on-going holobiont oxygen production measured within the same experiment (Stuhr et al. 2017) further supports these findings and highlights the severe consequences of thermal stress on proteins responsible for the generation of carbohydrates and cellular energy.

Symbionts – Adjustment of resource management and cell cycle

In order to maintain the cellular energy metabolism and provide carbon to downstream metabolic pathways during reduced photosynthesis, glucose produced by degradation of storages such as polysaccharides and lipids needs to be increasingly catabolized via glycolic processes and the tricarboxylic acid (TCA) cycle. Correspondingly, some enzymes that are typically taking part in carbohydrate, lipid and amino acid metabolism were elevated under chronic thermal stress. The DAPs included enzymes of different respiratory pathways and even enzymes involved in photorespiration such as the glyoxylate cycle (aconitase hydratase 2) (Kroth et al. 2008), which bypasses the CO_2-generating steps of the TCA cycle. The increase of acyl-CoA dehydrogenase, which initiates and catalyzes the rate-limiting step in the conversion of lipids to acetyl-CoA, showed an enhanced lipid catabolism. Besides, we found small GTPases, GTP- and ATP-binding proteins to have highly increased in abundance. These are involved in signal transduction, ion transport (e.g., Ca^{2+}-ATPase), but also play roles as sources of energy or activators of substrates in metabolic reactions and are used for protein synthesis and gluconeogenesis. Augmented degradation of lipids and carbohydrates through glycolysis and the

TCA cycle leads to elevated production of amino acids and other important biomolecules. As also cell cycle-related proteins increased, biosynthetic processes and cellular reorganization might have been supported, which likely required enhanced signal transmission, compound and energy supply. In diatoms grown under suboptimal condition of nutrient or iron limitation, alike shifts from proteins related to photosynthetic carbon fixation to proteins involved in cellular respiration (Luo et al. 2014) and ribosomal translation (Boyd et al. 2015) were observed. Jointly, the observed symbiont-associated DAPs in our study suggest that the stress response and degradation processes of the symbionts came along with the reduction of CO_2 concentrating mechanisms, leading to enhanced breakdown of energy storages and reorganization of other cellular constituents, possibly by recycling the remaining compounds through alternative metabolic pathways (Kroth et al. 2008).

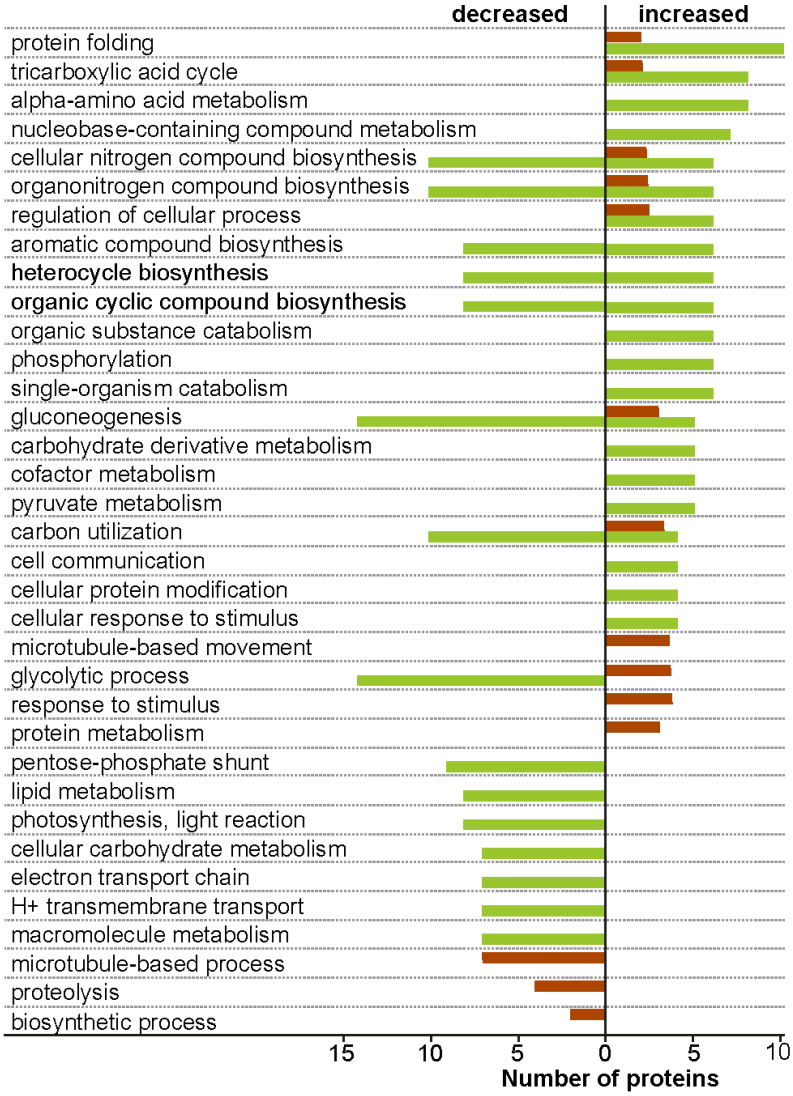

Figure 4-3. Counts of differently abundant proteins in Amphistegina gibbosa *in response to chronic thermal stress in the host (brown) and the symbiont (green) compartment grouped by biological process gene ontology terms. Position of bar indicates if proteins were more (right side) or less (left side) abundant than in the control.*

Host – Protein production, folding and degradation

Opposite to the symbionts, more than twice as many DAPs were enriched than reduced in the host compartment. The majority of the elevated proteins likewise included heat shock proteins and other chaperones or constituents involved in protein folding and quality control as well as proteolysis (Fig. 4-4b). These outcomes confirm the results of an Hsp70 immunoblotting study on the non-symbiotic benthic foraminifera *Ammonia tepida* (Heinz et al. 2012), which showed that elevated temperatures induce a significant increase in heat shock proteins in foraminifera, as it does under stressful conditions (Gethering and Sambrook 1992) in most organisms. Although foraminifera ceased to move or grow (Stuhr et al. 2017) lethal heats were not reached in our chronic-stress treatment, as then no foraminiferal Hsp70 would be detectable anymore (Heinz et al. 2012). Besides a variety of stress proteins (i.e., Hsp70/90 family, chaperonin, calreticulin, prohibitin and ubiquitin), a 26S protease regulatory subunit and caseinolytic peptidase (Clp), were elevated. These peptidases regulate intracellular protein levels as well as the turnover of defective proteins (Page and Di Cera 2008). They thereby contribute to maintaining the cellular proteostasis in selectively removing damaged or incorrectly folded proteins (Gottesman et al. 1990). Specifically the 26S proteasome is responsive to oxidative stress and was equally enhanced in anemones under heat stress (Oakley et al. 2017). This seems to be at odds with the observation that another serine peptidase (S10) was depleted, which specifically cleaves small peptides instead of full-length proteins (Page and Di Cera 2008). Its deficiency might indicate a shift of proteolysis from decomposing smaller biomolecule remains to concentrating on whole protein degradation in order to maintain homeostasis.

The stress induced boost of protein turnover was further indicated by enhanced cell cycle relate functions. Extreme increases in abundance were found in a RNA helicase (DEAD box polypeptide 46) and a translation initiation factor that are implicated in the alteration of RNA cellular processes necessary for protein synthesis, cellular growth and division. Furthermore, two signaling small GTPases (cell division control 42 and Ypt1) were increased. The former can interact with multiple regulators and effectors to activate a variety of cellular processes, mostly pathways leading to actin rearrangements and transcriptional inductions (Johnson 1999). Ypt1 regulates the trafficking of secretory vesicles from the endoplasmic reticulum (ER) to the Golgi, conducted by the trafficking particle complex (TRAPP) (Jones et al. 2000), of which one subunit was also elevated. Jointly, the augmentation of proteins involved in the translation, transport and modification of proteins, together with simultaneous elevated abundance of molecular chaperones and regulatory proteins, indicate an intensification of cell cycle related processes in the foraminifera that kept the cellular homeostasis of the host intact.

Host – Metabolic adjustments

Maintaining homeostasis in response to thermal change also requires shifts in metabolism to accommodate changes in the organism's energy requirements or of the flux of different metabolite classes. The strongest DAP decrease in the host was found for a phosphoethanolamine *N*-methyltransferase-like enzyme participating in the metabolism of glycerophospholipids, the main components of biological membranes. This is in contrast to heat shocked anemones that showed a strong increase in this enzyme (Oakley et al. 2017). A reason might be an inhibition of *S*-adenosyl-L-methionine (SAMe) synthesis through the methionine cycle, because enzymes depending on this primary donor of methyl in eukaryotic cells were depleted in both compartments. Another reduced enzyme (serine palmitoyltransferase) is essential for the biosynthesis of sphingolipids, which may serve vital functions in cell biology. The activity of this housekeeping enzyme is regulated in diverse ways and was suggested to be increased during apoptosis in response to certain types of stress (Hanada 2003), while the lowered abundance observed in our study might as well be due to deficiencies of essential constituents.

The up-regulated metabolic changes were more pronounced, including a wide range of proteins involved in core carbon metabolism. Assuming that significantly less photosynthate was released from the impacted photosymbionts to the host, metabolic needs have had to be met by adjusting pathways from mainly relying on carbohydrates supplied by the diatoms to digesting energy stored e.g., in lipid droplets. Similar to the symbiont compartment, an increase of cellular respiration was observed, specifically among enzymes catabolizing glycolysis (and gluconeogenesis), which represents a highly conserved response to cellular stress. Previous studies (Talge and Hallock 2003) on the ultrastructure of *A. gibbosa* describe significant declines of lipid bodies along with reduced numbers of viable symbionts during LBF bleaching. We therefore hypothesize, specifically with regard to the high energetic requirements for stress response and repair mechanisms, that elevated TCA cycle activity and digestion of lipids and other storages compensate for the photosynthate deprivation in order to meet the host's metabolic demands.

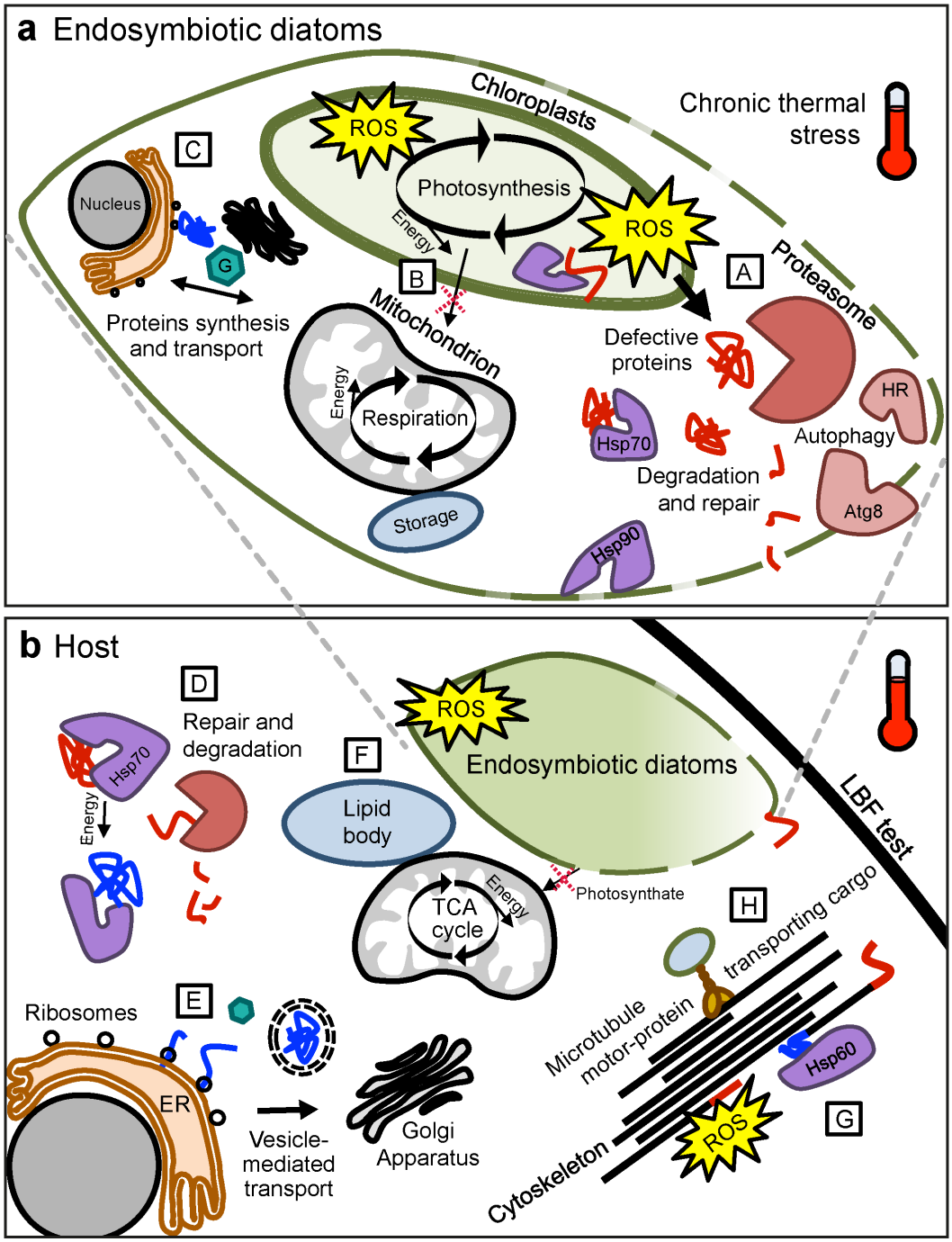

Figure 4-4. Chronic thermal stress induced the proposed cellular processes in the (a) photosymbionts and (b) the host cell of the large benthic foraminifera Amphistegina gibbosa. *(A) Malfunctioning photosystems in the diatom chloroplasts produce reactive oxygen species (ROS), which damage cellular compounds. Molecular chaperones (purple) such as Hsp70 and Hsp90 stabilize and repair defective proteins (red) or target them for degradation by the proteasome. Cell death-related proteins (rose) such as hypersensitive response (HR) and autophagy-related protein 8 (Atg8) disassemble cellular components. (B) The damaged photosystems produce less cellular energy and photosynthate. To compensate for this and meet metabolic requirements, alternative resources e.g., stored in form of lipids are catabolized by the mitochondria. (C) Cellular constituents are partly reorganized through enhanced ion transport and cellular signaling 'G' proteins in combination with augmented biosynthesis of proteins (blue), possibly recycling the degraded compounds. In the host (b), the ROS lead to damaged or incorrectly folded proteins (D), but cellular homeostasis is maintained by chaperone-modulated repair mechanisms and targeted protein degradation. (E) Cell cycle-related functions of the endoplasmic reticulum (ER), ribosomes and the Golgi such as protein synthesis and modification combined with increase in proteins responsible for transport and signal*

conduction facilitates enhanced protein turnover. (F) Energy resources stored in lipid bodies are metabolized, e.g., through the tricarboxylic acid (TCA) cycle, to meet the augmented metabolic needs necessary for these stress response mechanisms, despite diminished supply of photosynthate by the impaired symbionts. (G) The cytoskeleton is damaged by the ROS-induced stress (red ends), but increased amounts of chaperonins such as Hsp60 assist in folding and stabilization of new cytoskeletal proteins. (H) Microtubule motor-proteins such as dynein increase to enhance intracellular transport, which could enable the host foraminifera to enhance heterotrophic feeding, transport of (damaged) proteins or symbionts to their required location, and the reallocation of energy storages.

Host – Contradicting trends of cytoskeletal proteins

Actin and tubulin constitute the cytoskeleton and represent the majority of proteins found in foraminifera (Travis and Bowser 1986). Under chronic thermal stress, both were strongly depleted. These biomolecules fulfill diverse partly overlapping cellular functions (e.g., cell migration, adhesion and division). While actin builds filaments and plays a role in membrane trafficking, tubulin builds the microtubules that participate in the control of protrusive and contractile forces. Both compounds interact in multiple ways, such as in cell motility (Travis and Bowser 1986) i.e., movement of the holobiont within the vial, which accordingly is severely impaired in LBF by chronic thermal stress (Schmidt et al. 2011; Stuhr et al. 2017). Likewise, former cytological studies revealed deterioration of the host cytoplasm under combined stress of high temperature and light (Talge and Hallock 2003). Similar processes have likely occurred to a minor extent in our experiment. Actin filaments are highly sensitive to oxidative stress and were equally impacted in thermally shocked *Aiptasia* (Oakley et al. 2017). As in anemones, the combination of reduction in cytoskeletal proteins with increases in molecular chaperones and proteins responsible for translation and transport of new biomolecules indicate a replacement of the lost proteins.

Counter to the reduction of cytoskeleton-building proteins, proteins involved in microtubule-based movement (e.g., dynein) increased in response to chronic heat exposure. This suggests that the host raised its microtubule-motor activity in order to enhance cytoplasmic transportation of particles as would be necessary for more heterotrophic feeding or greater motility (Travis and Bowser 1986). Phototaxic studies have shown that LBF seek shade through reticulopodial locomotion when exposed to high light intensities (Zmiri et al. 1974), which might also be caused by high temperatures. Furthermore, it was recently shown that actin-mediated relocation of symbionts in LBF plays a key role in photoprotective mechanisms (Petrou et al. 2017), moving symbionts away from the high light causing photic stress. Another reason might be that, as suggested for corals (Wooldridge 2014), the importance of heterotrophic feeding in order to meet nutritional demands was increased during bleaching, requiring high cytoskeletal activity to collect and transport food particles into the endoplasm. Since specimens exposed to chronic stress showed severe bleaching, lowered chlorophyll *a* concentrations, and cease of

movements at the end of the experiment (Stuhr et al. 2017), we conclude that microtubule-activity was enhanced for intensification of intracellular transport, potentially enabling enhanced suspension feeding, transport of (damaged) proteins or symbionts targeted for degradation, or the reallocation of energy storages.

4.3 Discussion

Our dual-compartment quantitative proteomics approach sheds light into the complex mechanisms responsible for the repair, translation and degradation of cellular functions during different heat stress exposure scenarios. The general proteome response pattern is similar to the observed physiological parameters (Fig. 4-1), confirming that the presented novel approach worked for the analysis of small-sample amounts where both symbiotic compartments cannot be physically separated and the host has only low sequence coverage. As the outcomes of many physiological parameters are redundant if proteome data can be obtained, this implies recommendations concerning the selection of methods for biological studies on foraminifera. Additionally, our proteomics approach revealed the cellular mechanisms underlying these distinct reactions. The data denotes that the endosymbiotic diatoms were impacted more severely by elevated temperature than the host foraminifera. The symbiont proteome indicates reduced ability to photosynthesize, whereas the foraminifera show a signature of metabolic adjustment in favor of heterotrophy, compensating the less of symbiont-derived metabolites. This is in line with former studies suggesting that the host foraminifera is more resistant towards heat than their photosymbionts (Fujita et al. 2014; Stuhr et al. 2017).

We confirm the physiological experiment in that the strongest response is seen in chronic stress, whereas fluctuating water temperatures slightly impacted the photosymbionts, resulting in elevated activity in the host. The molecular mechanisms underlying the strong chronic-stress response involve degradation of defective compounds and repair of cellular damage, which is likely caused by ROS (Fig. 4-4). The thermal stress severely obstructed the functioning of the symbiotic diatoms, as observed in the detected photopigment loss and extensive bleaching. Increases in cell death and repair-related proteins indicate the disruption of proteostasis, at the same time and carbon concentrating mechanisms and transport of their products diminished. Foraminifera-associated proteins responsible for microtubule-based movement were strongly increased, along with molecular chaperones and holobiont total antioxidant capacities (Stuhr et al. 2017). Thus, remaining resources were likely distributed from cell migration and growth towards stress response and the cellular reorganizations of the host. During the constant heat exposure of 30 days, these protective mechanisms, e.g., unfolded protein response and rapid protein turnover, apparently facilitated the LBFs survival. Key to this relatively high stress resistance could be the high nutritional flexibility of

foraminifera, feeding on the photosynthate of their endosymbionts, storing energy in lipid droplets and the possibility to additionally feed heterotrophic, and should therefore be studied in more detail. Furthermore, bleaching in LBF has often been reported in combination with other impacts or diseases (such as reproductive dysfunction, infestations and malformations) (Williams et al. 1997; Hallock et al. 2006). These likely represent secondary effects or long-term consequences of resource redistribution. Such potential trade-offs deserve further investigation by targeted proteomic studies.

This study demonstrates the applicability of label-free proteomics on a non-model symbiotic organism and illustrates that the presented method offers novel opportunities to simultaneously study both compartments of photosymbiotic organisms, providing detailed insights into proteome responses and their effects on molecular functions. The successful application of the approach on a group with unusually poor molecular database coverage indicates that proteome analysis as implemented in this study may help to reveal the mechanisms of ecological response, biotic interactions and ecosystem-relevant functions in a range of similar organisms.

4.4 Methods

4.4.1 Thermal stress experiment

The experimental thermal-stress treatments implemented in this study were discussed in detail in previous work (Stuhr et al. 2017). *Amphistegina gibbosa* were collected in 20 m depth at Tennessee Reef in the Florida Keys (24°45'8.33"N, 80°45'26.33"W). The LBF were brought to the laboratory in Bremen, Germany, and maintained at established culture conditions (25.5°C, 5 - 10 µmole photons $m^{-2}s^{-1}$ on a 12-h light/dark cycle) for three weeks prior to the experiment (Fig. 4-S1a). During the entire time they were kept in 18-l aquaria filled with synthetic seawater (salinity 35.5, Tropic Marin Sea Salt, Germany) and equipped with a temperature sensor and a titanium heating rod to automatically control the thermal conditions, as well as an aquarium pump to circulate the water to simulate natural flow conditions (all from Aqua Medic, Germany). Temperature was logged constantly (HOBO Pendant, Germany), while salinity, pH and temperature were manually measured every other day.

Within each of the 12 independent, randomly allocated aquaria, ten *A. gibbosa* specimens were kept in a glass vial, covered with a 400 µm-nylon mesh, which allowed the water to circulate into the vial, while keeping the LBF inside. After three weeks of acclimation, the experiment ran for 30 days with three replicates of each of the four different thermal treatments simulating: (i) no thermal stress at culture conditions to serve as control; (ii) a single 3-day thermal stress event (up to 32°C) followed by stable control condition; (iii) four episodic 3-day thermal stress events

(up to 32°C) intermitted by six days at control conditions; and (iv) chronic thermal stress at 32°C (Fig. 4-S1b). The response variables of *A. gibbosa* and their photosymbionts (growth, motility, respiration, photosynthesis, coloration, chlorophyll *a* content) were documented on days 0, 3, 12, 21, and 30 in order to monitor the temporal variations in their physiological performance (Stuhr et al. 2017).

At the start of the experiment, i.e., after acclimation, five subsamples of ten specimens each were taken as initial controls. By aid of a fine paintbrush, the specimens were very shortly placed on a filter paper to remove the circumjacent water, directly transferred into cold 1.5 ml Protein LoBind tubes (Eppendorf, Germany), and immediately frozen at –80°C until further processing. The same procedure was performed with all samples at the end of the experiment.

4.4.2 Proteome analysis

The following chemicals were obtained from Sigma Aldrich, Steinheim, Germany: anhydrous magnesium chloride ($MgCl_2$), guanidine hydrochloride (GuHCl), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3) and urea. Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were from Merck, Darmstadt. Sodium dodecyl sulfate (SDS) was bought from Carl Roth, Karlsruhe, Germany. Tris base was purchased from Applichem Biochemica, Darmstadt, Germany. Dithiothreitol (DTT), EDTA-free protease inhibitor (Complete Mini) tablets were obtained from Roche Diagnostics, Mannheim, Germany. Sequencing grade-modified trypsin was bought from Promega, Madison, WI USA. All chemicals for ultra-pure HPLC solvents such as formic acid (FA), trifluoroacetic acid (TFA) and acetonitrile (ACN) were obtained from Biosolve, Valkenswaard, the Netherlands.

Eight specimens were pooled per experimental condition and biological replicate in 1.5 ml Eppendorf tubes, resulting in 12 sample pools. After addition of 100 µl lysis buffer (LB), comprised of 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% SDS and Complete Mini, lysis was carried out by mechanical grinding (Fig. 4-S1c). After storage on ice for 30 minutes, samples were clarified by centrifugation at 4°C and 10,000 rcf for 10 min.

Protein concentration was estimated based on reference samples of which the exact protein concentration was determined by amino acid analysis as previously described (Cohen and Michaud 1993; Shindo et al. 1997). Cysteines were reduced by the addition of DTT to a final concentration of 10 mM and incubation at 56°C for 30 min. Subsequently free thiol groups were alkylated with 30 mM IAA at room temperature (RT) for 30 min in the dark.

Buffer exchange and proteolysis were carried out by an adapted filter-aided sample preparation (Manza et al. 2005; Wisniewski et al. 2009) workflow. Lysates corresponding to an approximated protein concentration of 9 µg were diluted 5-fold by addition of freshly prepared (Kollipara and Zahedi 2013) 8.0 M Urea / 100 mM Tris-HCl (pH 8.5) and transferred onto the centrifugal device (PALL Nanosep, 30 kDa cutoff). Centrifugation was carried out at RT for 30 min at 13,500 rcf and all following centrifugation steps were performed under the same conditions for 15 min. Three wash steps were carried out with 100 µl of 8.0 M Urea / 100 mM Tris-HCl (pH 8.5). To exchange the buffer, the centrifugal devices were washed three times with 100 µl of 50 mM NH_4HCO_3 (pH 7.8). To the concentrated proteins, 100 µL of proteolysis buffer comprising trypsin (1:20 w/w ratio of protease to substrate), 0.2 M GuHCl and 2 mM $CaCl_2$ in 50 mM NH_4HCO_3 (pH 7.8) was added and samples were incubated at 37°C for 14 h. Digested peptides were recovered by centrifugation followed by consecutive washing steps with 50 µL of 50 mM NH_4HCO_3 and 50 µL of ultra-pure water. The digestion was stopped by addition of 20 µl of 10% TFA. Digests were desalted using SPEC C18, 4 mg sorbent (Agilent) as per manufacturers' instructions, and quality-controlled as described previously (Burkhart et al. 2012).

First, aliquots of each sample corresponding to ~1 µg of peptides were analyzed on a nano-LC-MS system in order to compensate for systematic errors derived e.g., from the protein concentration estimation. Thus, the sample amounts were corrected based on the alignment of total ion chromatograms to warrant identical starting material prior to actual LC-MS analysis. After normalization of amounts, all twelve samples (each 1 µg) were analyzed using an Ultimate 3000 nano RSLC system coupled to a Q Exactive HF mass spectrometer (both Thermo Scientific, Fig. 4-S1d). Peptides were preconcentrated on a 100 µm × 2 cm C18 trapping column for 10 min using 0.1% TFA with a flow rate of 20 µL/min followed by separation on a 75 µm x 50 cm C18 main column (both PepMap RSLC, Thermo Scientific) with a 120 min LC gradient ranging from 3 - 35% of buffer B: 84% ACN, 0.1% FA at a flow rate of 250 nL/min. The Q Exactive HF was operated in data-dependent acquisition mode and MS survey scans were acquired from m/z 300 to 1,500 at a resolution of 60,000 using the polysiloxane ion at m/z 371.101236 as lock mass (Olsen et al. 2005). Isolation of precursors was performed by the quadrupole with a window of 0.4 m/z. The fifteen most intense signals (Top15) were subjected to higher energy collisional dissociation with a normalized collision energy of 27% at a resolution of 15,000, taking into account a dynamic exclusion of 12 s. Automated gain control target values were set to 3×10^6 for MS and 5×10^4 MS/MS. Maximum injection times were 120 ms and 250 ms, respectively. Precursor ions with charge states of +1, > +5 or unassigned were excluded from MS/MS analysis. The 'underfill' ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 5%, which corresponds to a

minimum precursor intensity of 2.5 × 10^3 to trigger a MS/MS scan. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD008015.

4.4.3 Data analysis

Data analysis for label free quantification was performed using the Progenesis LC-MS software (version 3.0.6039.34628) from Nonlinear Dynamics (Newcastle upon Tyne, U.K.) and the statistical software R, version 3.3.1 (R Core Team 2016). Biological triplicates from all treatments at the end of the experiment were compared to the start condition, and for further insights, biological triplicates of single-, episodic- and chronic-stress treatments were compared to the control (Fig. 4-S1e).

Raw MS data was imported into Progenesis and aligned to one automatically selected LC-MS reference file. After peak picking, MS/MS spectra were exported as peak list and identification of proteins and peptides was performed by the PEAKS Studio software suite, version 7.5, *de novo* to SPIDER (Zhang et al. 2012). Searches were performed in a decoy-fusion manner against a concatenated database comprising publicly available protein and nucleotide sequences of foraminifera as well as diatoms (Bacillariophyta) from the NCBI database on 2016/03/15. Protein sequence databases were used as such, while nucleotide sequences were translated in all six reading frames prior to concatenation. Precursor mass tolerance was set to 10 ppm and fragment ion tolerance to 0.02 Da. Enzyme specificity was set as fully tryptic, with a maximum of two missed cleavages. For *de novo* and DB search, carbamidomethylation of cysteines was defined as fixed modification and oxidation of methionine as variable modification. A maximum number of two variable modifications per peptide was allowed for *de novo* and DB search, while for the PTM search all common modifications were allowed (485 in total) and the maximum number was set to three per peptide. Minimum *de novo* ALC for both PTM and SPIDER homology search was set to 15%. Peptide-level FDR was limited to 1% and proteins had to be identified with at least one unique peptide in order to be reported. Identifications meeting these criteria were re-imported into Progenesis to calculate the normalized abundances on the peptide-level.

Amino acid sequences of all proteins identified in the database search were uploaded in .fasta file format to the CD-HIT suite web server of the Weizhong Li Lab (Huang et al. 2010) in order to generate cluster of homologous protein sequences. Sequence identity cut-off was set to 0.7 and minimum alignment coverage for the longer sequence was set to 0.0. All other parameters were left as default. Normalized abundance-values of the peptides were used to calculate

protein cluster abundance (Fig. 4-S1f). Only peptides unique to a given protein cluster were used for quantification.
To identify statistically significant changes of protein abundance between the conditions, first the one-way between-subjects ANOVA was calculated. For regulated proteins, i.e., proteins with a *p*-ANOVA ≤ 0.05, Tukeys' HSD post hoc test was performed to determine the statistical significance for every individual condition compared to the control. Regulated protein clusters with a Tukeys' HSD post hoc test *p*-value ≤ 0.05 and a $\log_2$ fold change (FC) greater than 1 or below –1 were considered as significantly changed in abundance.

For all sequences that significantly changed in abundance in any of the thermal-stress treatments, we performed an additional gene ontology annotation using Blast2GO, version 4.1.5 (Conesa et al. 2005) to assess the protein identification coverage of our workflow and sequence databases (Fig. 4-S1g). All sequences were concatenated and searched against all eukaryotes in the non-redundant (nr) public NCBI database using blastp and a maximum E-value of 1.0×10^{-5} reporting only the three top Blast hits. This was followed by the mapping function to assign gene ontology (GO) terms to each sequence with Blast hits. The GO term annotations were evaluated by the Blast2GO annotation rule algorithm using the following criteria: E-value filter 1.0×10^{-6}, annotation cutoff 55 and GO weight 5. To complement the functional information of the proteins, an InterPro scan of these sequences was performed and GO information was merged. The resulting best Blast hits were adopted as sequence descriptions. If no specific protein names could be identified by the Blast search, available InterPro protein names were included. Based on the major molecular functions, biological processes and cellular component annotations, the proteins were grouped into different functional categories. These are not exclusive, but rather generic as many proteins can fulfill a multitude of functions and therefore might also fit into different categories. The entire annotation results can be found in the electronically available supplementary Table S4-4.

4.5 Acknowledgements

This project was funded by the Leibniz Association (SAW-2014-ISAS-2) awarded to AS and HW. Sampling was conducted under the Research Permit No. FKNMS-2015-026, issued to Pamela Hallock who is warmly acknowledged for her general support and assistance during fieldwork. Furthermore, BBL, LK and AS acknowledge the support by the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Regierende Bürgermeister von Berlin - incl. Wissenschaft und Forschung, and the Bundesministerium für Bildung und Forschung.

4.6 Supplementary materials

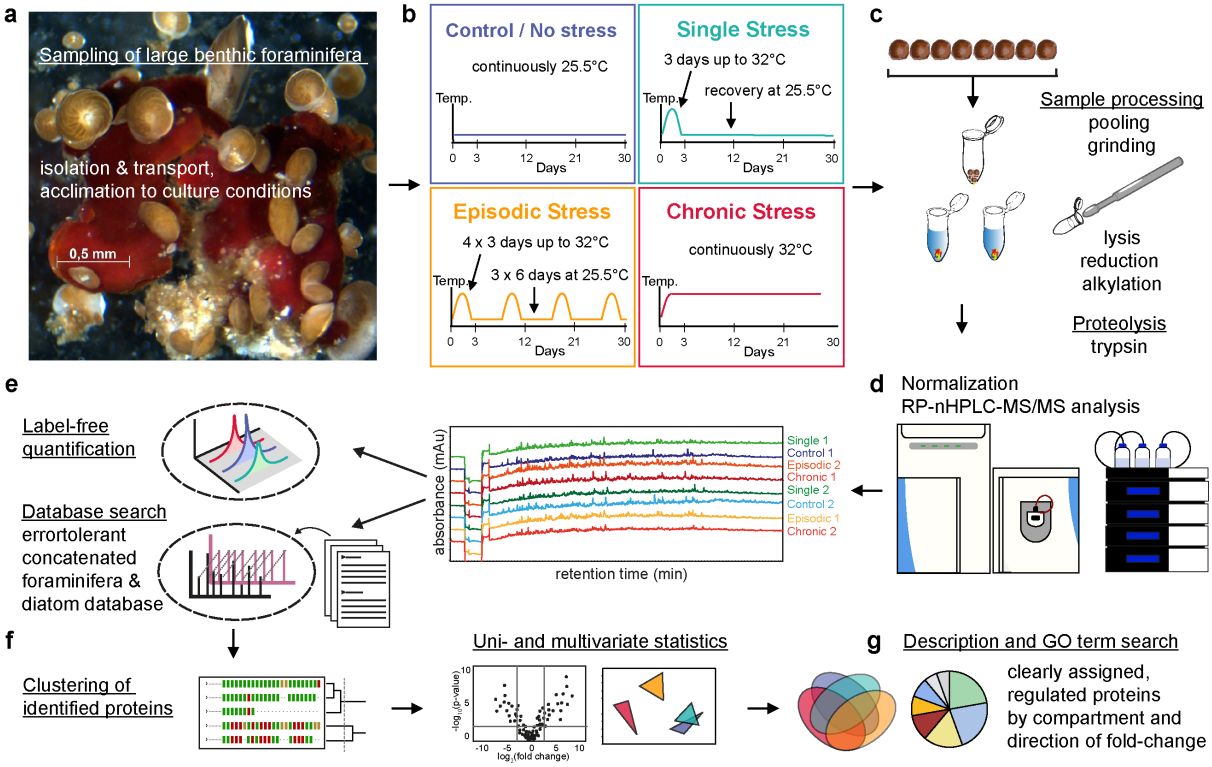

Figure S4-1. Schematic flowchart of the experimental setup and proteome analysis protocol of the thermal stress experiment on Amphistegina gibbosa. *(a) Specimens were collected from coral rubble in the Florida Keys at 18 m depth, isolated and acclimated for three weeks. (b) Over one month they were exposed to four temperature treatments, with three randomized replicate aquaria per treatment, mimicking different thermal stress scenarios. (c) From each treatment, 8 specimens were pooled, their protein content was extracted and clarified. Before protein digestion by trypsin, their concentrations were measured and samples were cleaned. (d) First, sample amounts of the desalted peptides were normalized by total ion current and then analyzed on a LC-MS/MS system. (e) Label-free quantification and protein identification as well as assignment to either host or symbiont compartment was followed by (f) homology-based protein grouping, calculation of protein cluster abundances and determination of statistically significant changes. (g) All protein clusters that were clearly 'regulated' were further characterized by annotation of protein descriptions and gene ontology terms.*

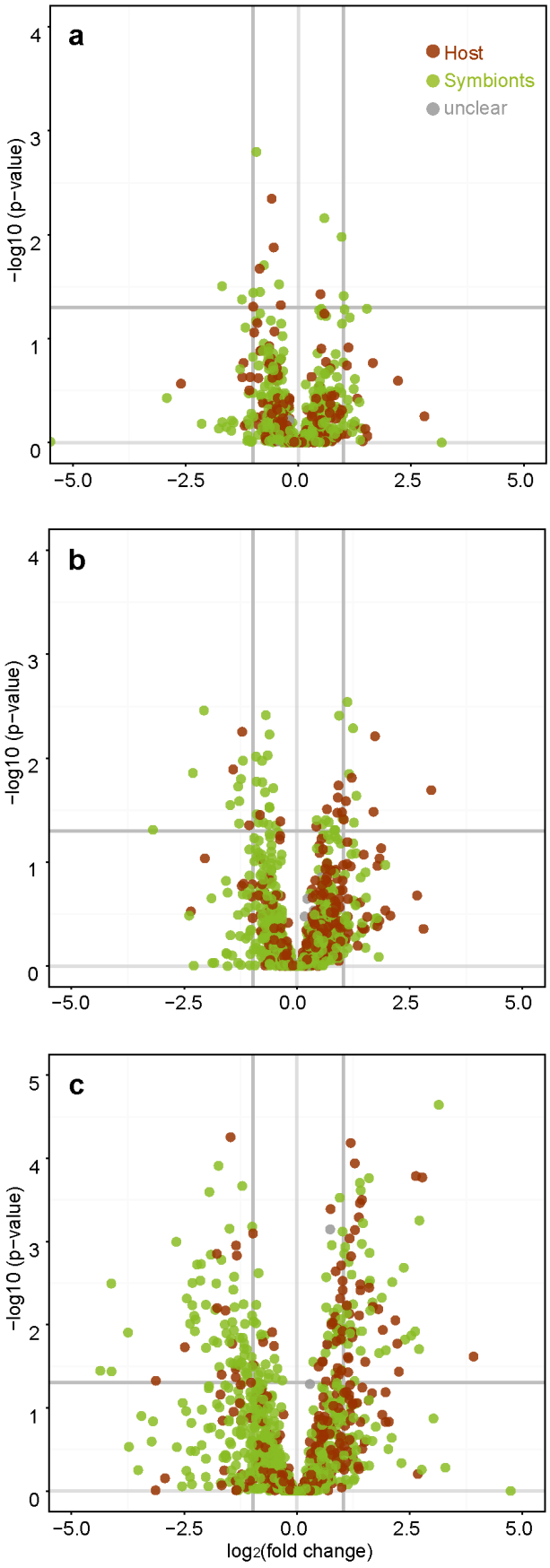

Figure S4-2. Volcano plots of log_2 fold changes versus –log10(p-values), representing the probability that the protein is significantly regulated, of all identified proteins in Amphistegina gibbosa *in response to (a) a single stress event, (b) episodic stress events, (c) and chronic stress (please note the different scale of the y-axis), compared to the control. Proteins above –log10(0.05) = 1.301 are considered significantly regulated where fold changes of above $–log_2(2) = 1$ specify increased and below $–log_2(0.5) = –1$ specify decreased abundances.*

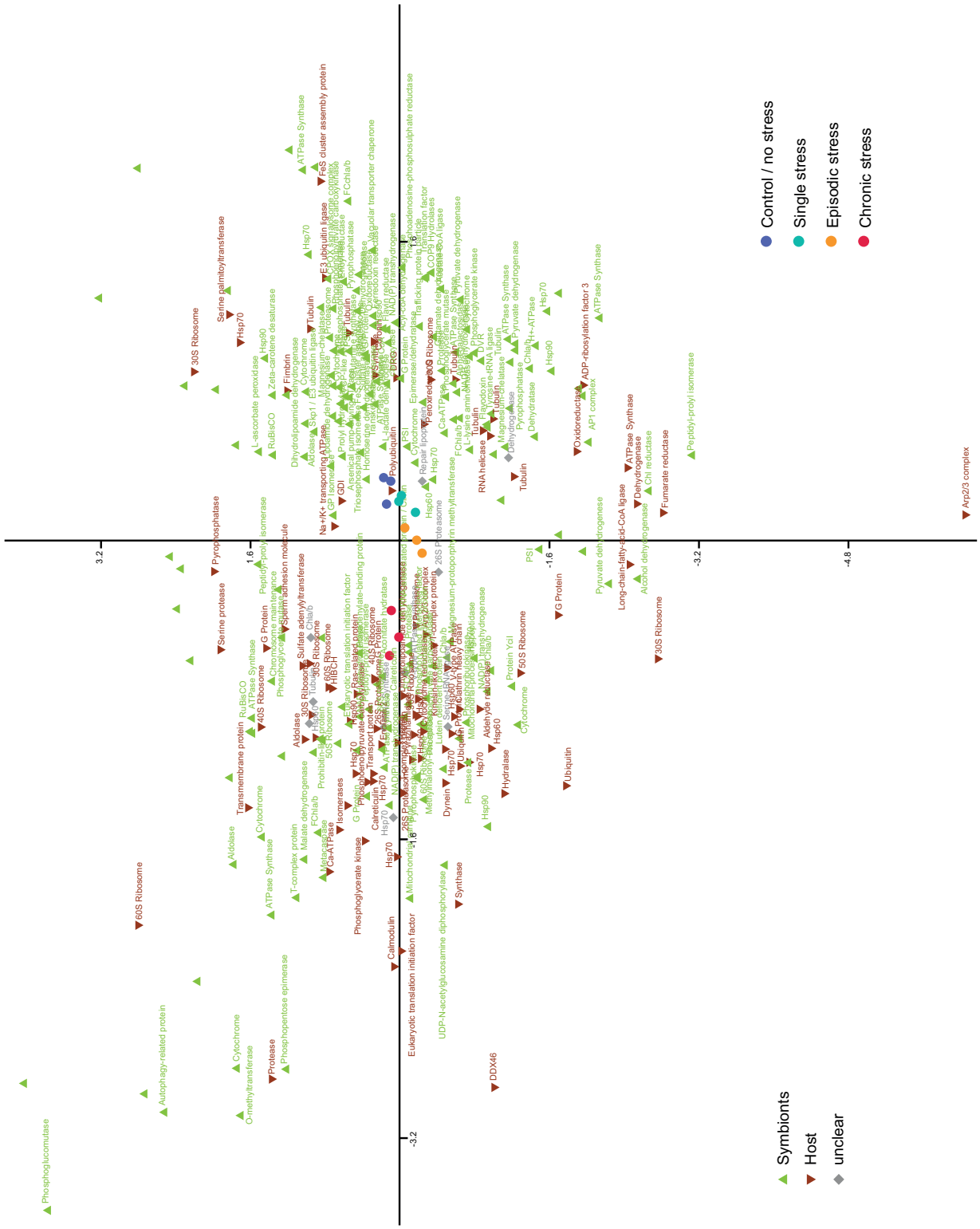

Figure S4-3. Correspondence analysis of relative protein abundances of all 294 regulated proteins in Amphistegina gibbosa *in response to a single short-term stress event (turquoise), episodic stress events (orange) or chronic thermal stress (red) compared to the control (blue), showing the distribution and descriptions of proteins (host = brown inverse triangles, symbiont = green triangles, unclear/both = grey diamonds) that characterize the directional changes between treatments with protein abundances as scaling variable. 65.8% are explained by variation along axis 1 and 11.2% by axis 2.*

Table S4-1. Symbiont-associated proteins that significantly changed in abundance in any of the treatments compared to the control.[a] Row colors indicate functional categories based on GO terms: purple = protein quality control and folding, dark blue = metabolism, green = photosynthesis, orange = biosynthesis and cell cycle, rose = proteolysis and autophagy, light blue = signalling, grey = transport. # = number of proteins in the cluster, UP = number of unique peptides. Log_2 fold changes are indicated by color (red = increase, blue = decrease). Significant results of Tukeys post hoc test are marked by asterisks: (*) p-value ≤ 0.05, (**) p-value ≤ 0.01 and (***) p-value ≤ 0.001.

Consensus Protein Description[a]	#	UP	Log_2 fold change single	episodic	chronic
ATPase synthase subunit beta	1	1	1.01*	1.16*	0.00
26S proteasome regulatory subunit	1	2	1.14	1.32*	0.75
peptidyl-prolyl cis-trans isomerase	1	3	0.69	1.24**	0.57
arginine biosynthesis protein ArgJ (mitochondrial)	1	2	0.96	1.12**	0.53
Clp protease A/B family / chaperonin	1	2	0.38	1.01*	1.13*
Small GTPase superfamily	2	2	0.77	1.30	3.14***
Probable serca-type calcium ATPase	1	1	-0.73	0.78	2.71***
band 7 domain-containing	1	1	-0.17	-1.24	2.71*
vacuolar transporter chaperone domain-containing	1	1	0.71	-1.87	2.62*
alcohol dehydrogenase	1	1	-1.13	-0.11	2.53*
member of the Clp large regulatory subunit	1	1	-1.07	1.03	2.40*
phosphomannose mutase	1	1	-0.12	-0.34	2.37**
histidinol dehydrogenase	1	1	0.66	1.09	2.11**
Tim10/DDP family zinc finger	1	1	0.39	0.05	1.97*
hypersensitive-induced response 1	1	2	-0.32	-0.02	1.88**
heat shock Hsp90	1	3	0.77	1.26	1.82*
arsenical pump ATPase	1	1	-0.27	0.54	1.68**
short chain acyl-CoA dehydrogenase	2	2	-0.01	0.89	1.62**
PP / NAD(P)-binding domain	1	1	0.27	0.77	1.62**
calreticulin	2	5	0.25	0.23	1.61**
heat shock protein hsc70	1	2	-0.11	0.43	1.60***
heat shock 70	2	16	0.34	0.78	1.59**
small GTPase superfamily	1	2	0.48	0.85	1.47***
aconitase hydratase 2	2	5	0.47	0.80	1.44*
delta-aminolevulinic acid dehydratase /	2	9	-0.23	-0.12	1.44**
heat shock protein/chaperone Hsp70	5	52	0.29	0.70	1.42***
prohibitin-like protein	1	2	0.67	0.55	1.41*
phosphorylated CTD-interacting factor 1	1	1	-0.17	0.39	1.39***
phosphoenolpyruvate carboxykinase	1	1	0.27	0.42	1.28*
tyrosyl-tRNA synthetase	1	1	0.31	0.57	1.28*
methylmalonyl-CoA mutase	2	3	0.46	0.90	1.25**
glyceraldehyde-3-phosphate dehydrogenase	6	17	0.50	0.70	1.21**
cyclin dependent kinase	1	1	0.54	0.34	1.18*
autophagy-related Atg8, ubiquitin-like	2	3	-0.41	-0.21	1.18*
protein of unknown function DUF760	1	1	0.28	-0.25	1.15**
canine-like Rab-type small GTPase	1	2	0.26	0.37	1.11**
plastid lipid-associated protein	1	1	0.51	0.51	1.11**
isochorismatase hydrolase	1	1	-0.06	0.32	1.07**

chaperone protein dnaK / Hsp70-type	4	34	0.37	0.94	1.05**
dihydrolipoyl dehydrogenase	4	7	0.61	0.83	1.03*
NAD-dependent malic enzyme	1	1	-0.05	0.50	1.01*
luminal binding protein / Hsp70 family	1	3	0.03	0.48	1.01***
cyclophilin-type peptidyl-prolyl isomerase	1	2	0.45	0.85	1.01*
LOV domain-containing protein	1	1	-1.25*	-0.69	-1.03
fucoxanthin chlorophyll a/c	1	1	-1.69*	-0.55	-1.30
glycoside hydrolase, family 65	1	1	-0.56	-1.20*	-0.14
glycoside hydrolase, family 65	1	2	-0.85	-1.24*	-0.25
catalytic activity	2	3	-0.65	-1.28*	0.02
histidine phosphatase superfamily, clade-1	1	1	-0.94	-1.28*	-1.23*
biotin- / acetyl-CoA carboxylase	1	2	-0.66	-1.30*	-1.81**
NADH dehydrogenase subunit 11 / RuBisCO small	1	1	-0.33	-1.48*	-4.12**
phospholipid methyltransferase / phosphoglycerate	1	1	-0.29	-2.06**	-6.57***
HopJ type III effector protein	1	1	-1.17	-2.31*	-2.02*
aldose 1-/glucose-6-phosphate 1-epimerase	1	1	-1.01	-3.19*	-4.36*
phosphoglycerate mutase / chlorophyll a/b binding	1	2	-0.31	-0.50	-1.00*
plastid lipid-associated protein / fibrillin conserved	1	1	-0.35	-0.21	-1.10*
1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate	1	5	-0.03	-0.68	-1.11*
phosphoglycerate kinase	4	37	-0.33	-0.57	-1.11*
magnesium-protoporphyrin IX methyltransferase	1	4	-0.23	-0.34	-1.12*
RuBisCO large subunit, ferredoxin-like	1	1	-0.68	-0.91	-1.15*
transketolase / phosphatidyl-N-methylethanolamine/N-methyltransferase	6	70	-0.44	-0.56	-1.15**
protoporphyrin IX magnesium chelatase, subunit H	2	4	-0.33	-0.64	-1.17*
ATP synthase subunit beta	3	46	0.43	0.27	-1.17*
cytosolic class II aldolase	1	2	-0.42	-0.52	-1.17*
cytochrome b6/f complex, subunit IV	1	1	-0.41	-0.90	-1.18**
dihydrolipoamide acetyl transferase	1	4	-0.56	-0.33	-1.20*
structural maintenance of chromosomes protein	1	1	-0.16	0.15	-1.20*
UDP-glucose-	4	7	-0.43	-0.61	-1.21***
RuBisCO large	6	84	0.35	0.06	-1.23*
photosystem I iron-sulfur center	2	9	-0.66	-0.72	-1.27*
2-oxoacid dehydrogenase acyltransferase	1	1	-0.05	-0.74	-1.28*
RuBisCO small subunit	4	32	-0.17	-0.60	-1.30*
transketolase-like	2	15	-0.32	-0.62	-1.34*
50S ribosomal protein / cytochrome b6	5	10	0.14	-0.22	-1.38**
precursor of dehydrogenase pyruvate	4	15	-0.30	-0.57	-1.39**
V-type proton ATPase subunit	1	1	-1.00	-0.84	-1.40*
cytochrome b6-f complex iron-sulfur subunit	2	12	0.31	-0.03	-1.41*
oxidoreductase / myo-inositol 2-dehydrogenase	2	10	-0.26	-0.74	-1.41**
acetyl-CoA carboxylase	3	31	-0.30	-0.25	-1.44**
phosphoglycerate mutase	1	3	-0.09	-0.95	-1.46*
cytochrome C peroxidase	1	7	-0.24	-0.13	-1.48*
NAD(P)	1	3	0.08	-0.61	-1.49***
cytochrome b6-f complex subunit IV	1	5	0.12	-0.20	-1.49*
dihydrolipamide S-acetyltransferase	1	2	-0.06	-0.44	-1.56*

cation transporting ATPase	2	5	-0.32	-0.52	-1.60*
ferredoxin--NADP reductase / phosphoglucose	9	44	-0.29	-0.57	-1.67**
dihydrolipoamide acetyl transferase	1	1	-0.23	-0.62	-1.72*
apospory-associated / ferredoxin--NADP reductase	1	3	-0.33	-0.65	-1.74***
fucoxanthin chlorophyll a/c protein	2	26	-0.34	-0.70	-1.76*
ATP synthase / precursor of ATPase gamma	3	7	-0.10	-0.28	-1.79**
clavaminate synthase	1	1	-0.16	-1.06	-1.84*
transhydrogenase / fructose-biphosphate aldolase	1	4	-0.20	-0.58	-1.91**
fructose-1,6-bisphosphate aldolase	2	21	-0.11	-0.77	-1.94***
lutein deficient 1-like protein	1	1	-0.77	-0.85	-2.02**
cytosolic class II aldolase	2	4	-0.45	-0.85	-2.13**
small ribosomal S1 / photosystem	1	3	0.01	-0.31	-2.14**
pyridine nucleotide-disulphide oxidoreductase	1	1	0.07	-0.40	-2.21**
ATP synthase subunit delta / plastid thylakoid	1	2	-0.41	-0.69	-2.25**
alanine dehydrogenase/pyridine nucleotide	1	2	0.13	-0.51	-2.26*
cytochrome b6-f complex iron-sulfur subunit /	1	1	-0.35	-0.34	-2.28**
glucose-6-phosphate isomerase	2	4	-0.44	-0.74	-2.32**
lipoamide dehydrogenase	1	9	-0.08	-0.59	-2.32**
ATP synthase beta subunit	7	84	-0.12	-0.43	-2.37**
ATP synthase b'	1	8	-0.39	-0.89	-2.44**
ATP sulfurylase	2	14	-0.24	-0.89	-2.68**
lipoamide dehydrogenase	1	1	-0.37	-0.81	-3.74*
HopJ type III effector protein	2	3	-0.06	-1.57	-4.11*

Table S4-2. Host-associated proteins that significantly changed in abundance in any of the treatments compared to the control. [a] Row colors indicate functional categories based on GO terms: yellow = cytoskeleton and microtubule-based processes, purple = protein quality control and folding, dark blue = metabolism, orange = biosynthesis and cell cycle, rose = proteolysis and autophagy, light blue = signalling, grey = transport. # = number of proteins in the cluster, UP = number of unique peptides. Log_2 fold changes are indicated by color (red = increase, blue = decrease). Significant results of Tukeys post hoc test are marked by asterisks: (*) p-value ≤ 0.05, (**) p-value ≤ 0.01 and (***) p-value ≤ 0.001.

Consensus Protein Description[a]	#	UP	Log_2 fold change		
			single	episodic	chronic
actin-related protein 2 (Arp2/3 complex)	1	3	2.21	2.98*	0.69
methylmalonyl-CoA mutase	1	1	1.08	1.73**	1.24
fumarate reductase	1	3	0.10	1.21*	-0.20
long-chain-fatty-acid-ligase	1	2	0.20	1.09*	0.14
amidohydrolase 2	1	1	0.96	1.70*	1.90*
chaperonin GRoEL / chaperonin 60	1	8	0.63	1.04*	1.40**
DEAD box polypeptide 46	1	1	1.43	2.81	3.92*
caseinolytic peptidase (ClpA/B family)	1	1	0.35	0.75	2.78***
eukaryotic translation initiation factor 4E type	1	2	0.92	1.48	2.64***
prohibitin	1	2	0.95	1.47	2.26*
ubiquitin / ribosomal protein Cep52 fusion	2	2	1.65	1.78	2.23*
hypothetical protein, kinesin-like	1	1	0.14	1.14	2.18**
phosphoglycerate kinase	1	1	0.44	0.77	1.80**
Hsp70	1	1	0.27	0.81	1.68**
trafficking particle complex subunit 3	1	1	0.43	0.55	1.60**
dynein, heavy polypeptide 5	1	1	0.36	0.84	1.51*
Hsp90 family	1	1	0.20	0.75	1.41**
phosphoenolpyruvate carboxykinase	2	4	0.08	0.53	1.40***
malic enzyme	1	1	0.49	0.57	1.38**
chaperone DnaK / Hsp70	2	7	0.38	0.92	1.37***
calreticulin/calnexin	1	3	0.26	0.53	1.29***
pyrazinamidase/nicotinamidase	1	1	0.51	0.67	1.28***
Heat shock protein 70C	1	2	0.21	0.43	1.23*
Rac GTPase / cell division control 42	1	2	0.48	0.58	1.21*
heat shock 60	1	2	0.07	0.67	1.20**
heat shock 70	2	18	-0.22	0.50	1.19***
GTP-binding Ypt1 / small GTPase superfamily	1	4	-0.20	0.36	1.17***
actin family	1	3	0.42	0.59	1.16**
26S protease regulatory subunit 6b	1	1	0.22	0.52	1.14*
aldo-keto oxidoreductase / alcohol dehydrogenase	1	1	0.42	0.90	1.14*
Hsp70	1	2	-0.07	0.67	1.13*
enolase 2	1	12	0.20	0.45	1.11**
ubiquitin hydrolase	1	3	0.36	0.41	1.02**
cytoplasmic dynein light chain	1	2	-0.07	0.10	1.01**
fructose-bisphosphate aldolase	1	5	0.12	-0.11	1.01*
hydrogen-translocating pyrophosphatase	1	1	-1.00*	-0.81	-0.28
serine carboxypeptidase S10	1	1	-0.91	-1.06*	-1.43*

actin family	1	1	-0.86	-1.22**	-1.78**
serine palmitoyltransferase 1	1	1	-0.34	-1.41*	-1.77**
serine carboxypeptidase S10	1	2	-0.46	-0.32	-1.02*
tubulin alpha-3	1	8	-0.40	-0.36	-1.33**
beta-tubulin	5	73	-0.20	-0.05	-1.35**
tubulin alpha-4	2	4	-0.23	-0.27	-1.35**
calponin homology domain	1	1	-0.98	-0.71	-1.36*
beta tubulin	1	2	-0.59	-0.37	-1.47***
coronin	1	1	-0.19	-0.61	-1.58**
tubulin	1	1	-0.41	-0.85	-1.67*
ATPase, dynein-related	1	1	-0.58	-0.99	-2.49*
phosphoethanolamine N-methyltransferase-like	1	1	-0.02	-2.04	-3.13*

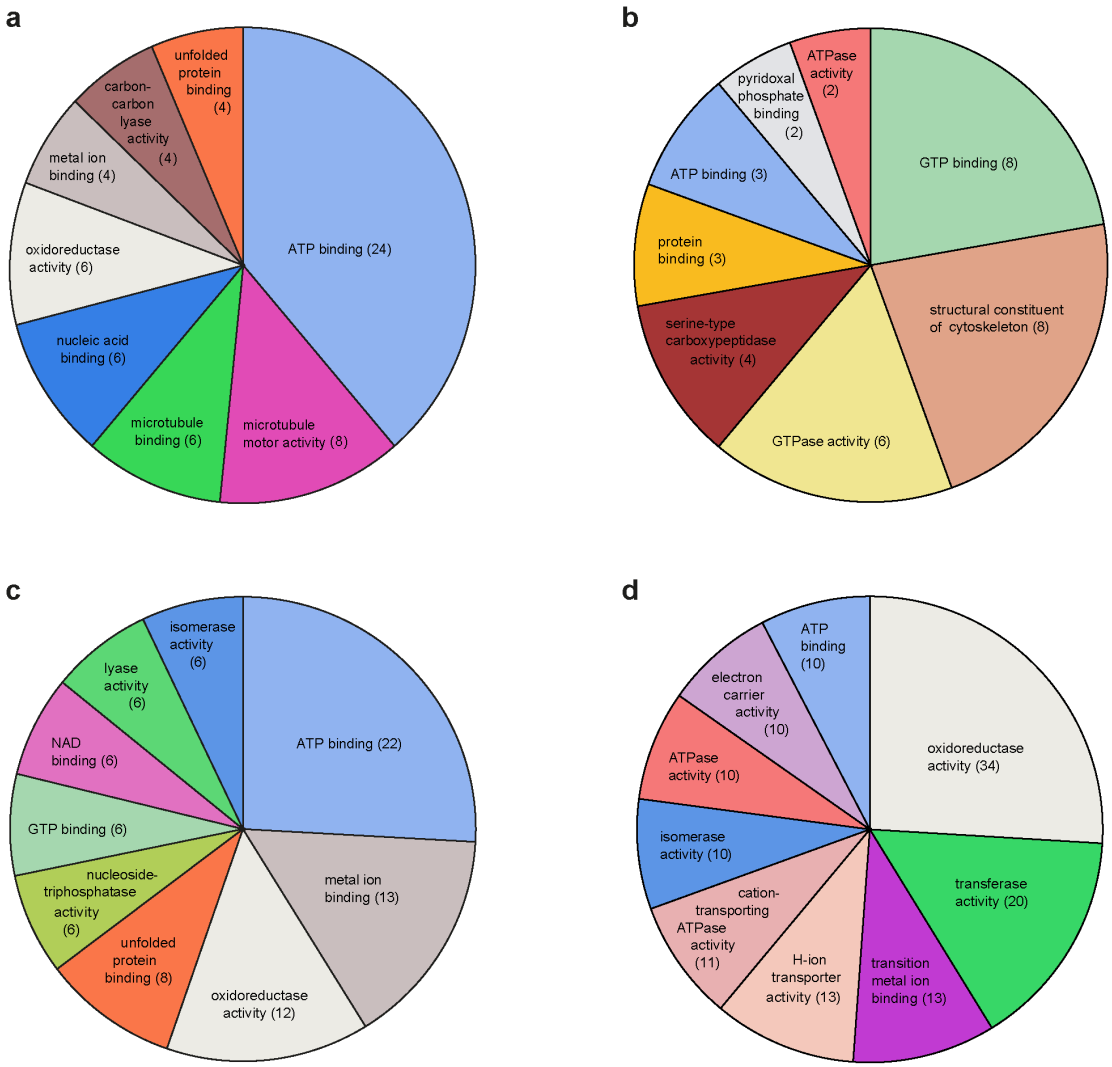

Figure S4-4. Sequence distributions of molecular function annotations of differently abundant proteins in Amphistegina gibbosa *in response to chronic thermal stress. Protein count charts are arranged by compartment: (a) and (b) are host-associated, (c) and (d) symbiont-associated, as well as by direction of change: (a) and (c) increased, while (b) and (d) decreased compared to the control. Numbers in brackets indicate protein sequence counts of the given gene ontology (GO) function.*

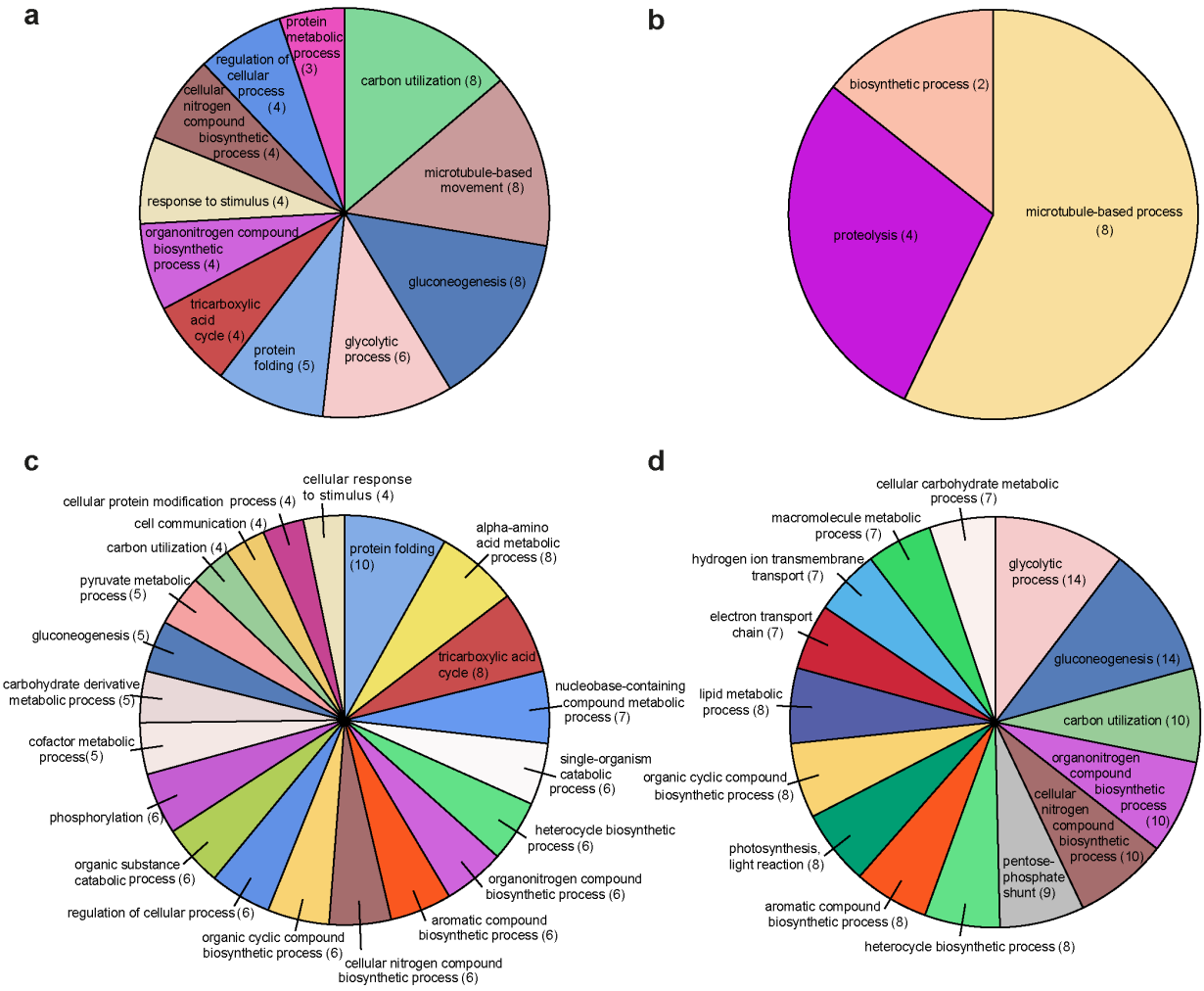

Figure S4-5. Sequence distributions of biological process annotations of differently abundant proteins in Amphistegina gibbosa *in response to chronic thermal stress. Charts are arranged by compartment: (a) and (b) are host-associated, (c) and (d) symbiont-associated, as well as by direction of change: (a) and (c) increased, while (b) and (d) decreased compared to the control. Numbers in brackets indicate protein sequence counts of the given GO process.*

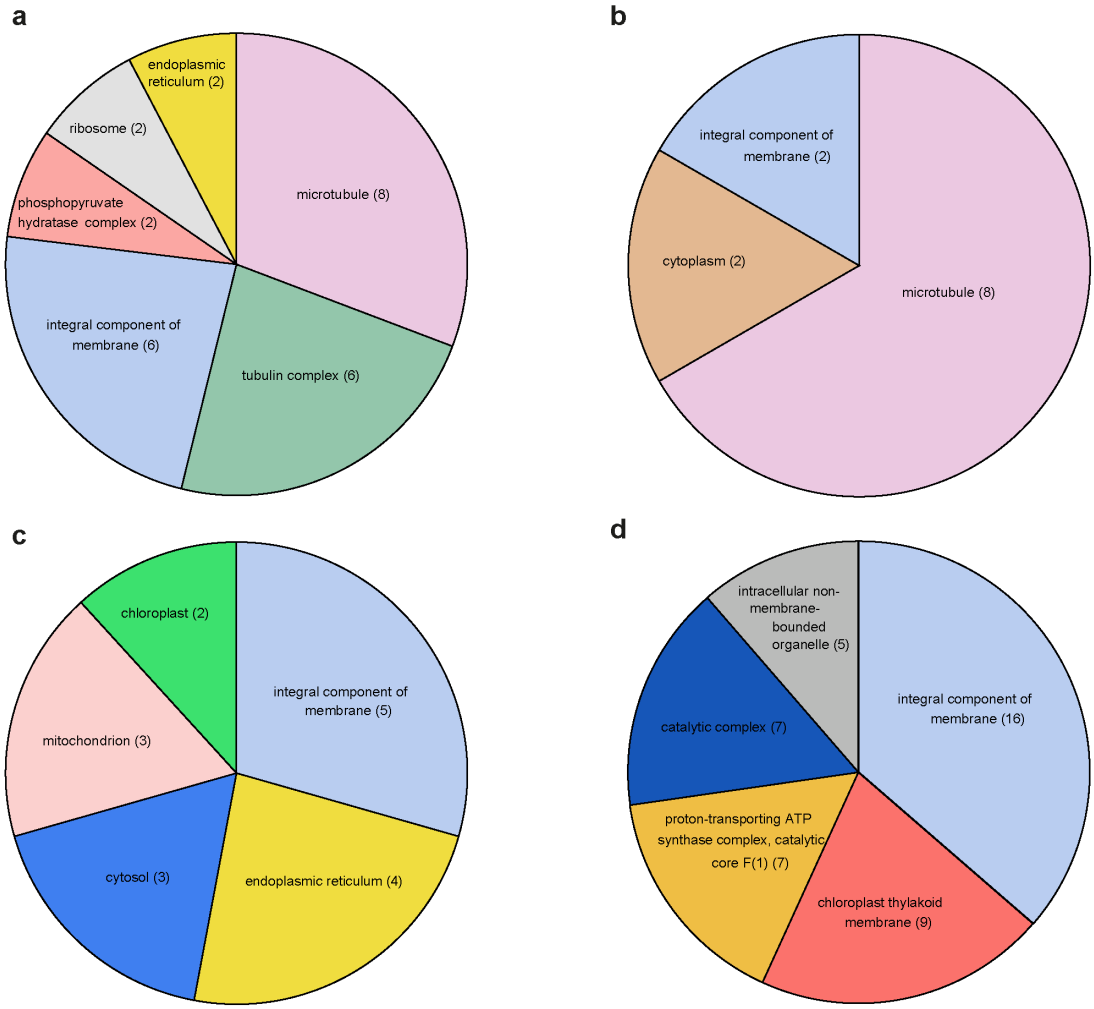

Figure S4-6. Sequence distributions of cellular component annotations of differently abundant proteins in Amphistegina gibbosa *in response to chronic thermal stress. Protein count charts are arranged by compartment: (a) and (b) are host-associated, (c) and (d) symbiont-associated, as well as by direction of change: (a) and (c) increased, while (b) and (d) decreased compared to the control. Numbers in brackets indicate protein sequence counts of the given GO component.*

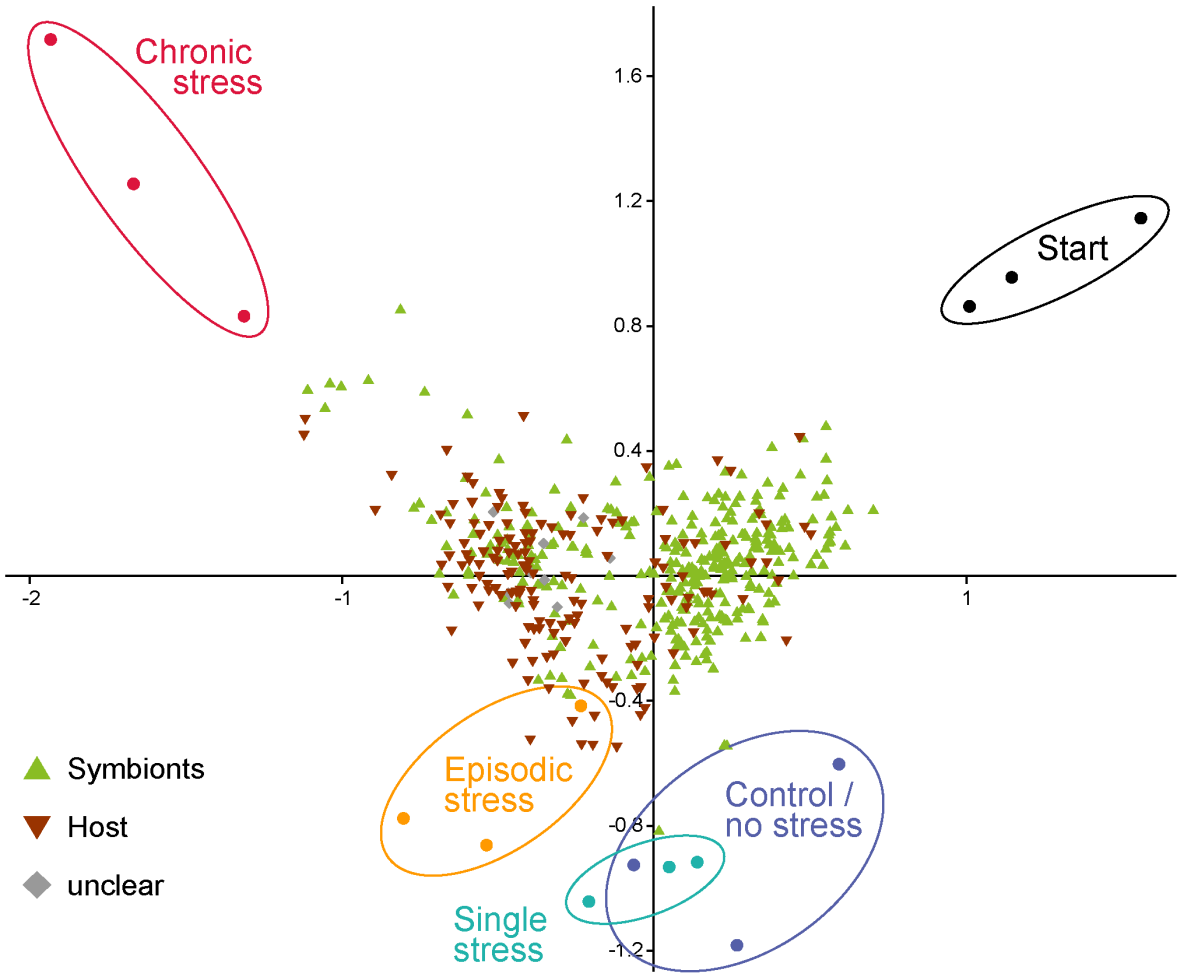

Figure S4-7. Correspondence analysis of relative protein abundances of all 491 regulated proteins in Amphistegina gibbosa *compared to the start (black) in response to the control treatment (blue), a single short-term stress event (turquoise), episodic stress events (orange) or chronic thermal stress (red), showing the distribution of proteins (host = brown inverse triangles, symbiont = green triangles, unclear/both = grey diamonds) that drive the directional changes between treatments. 55.9% are explained by variation along axis 1 and 17.4% by axis 2.*

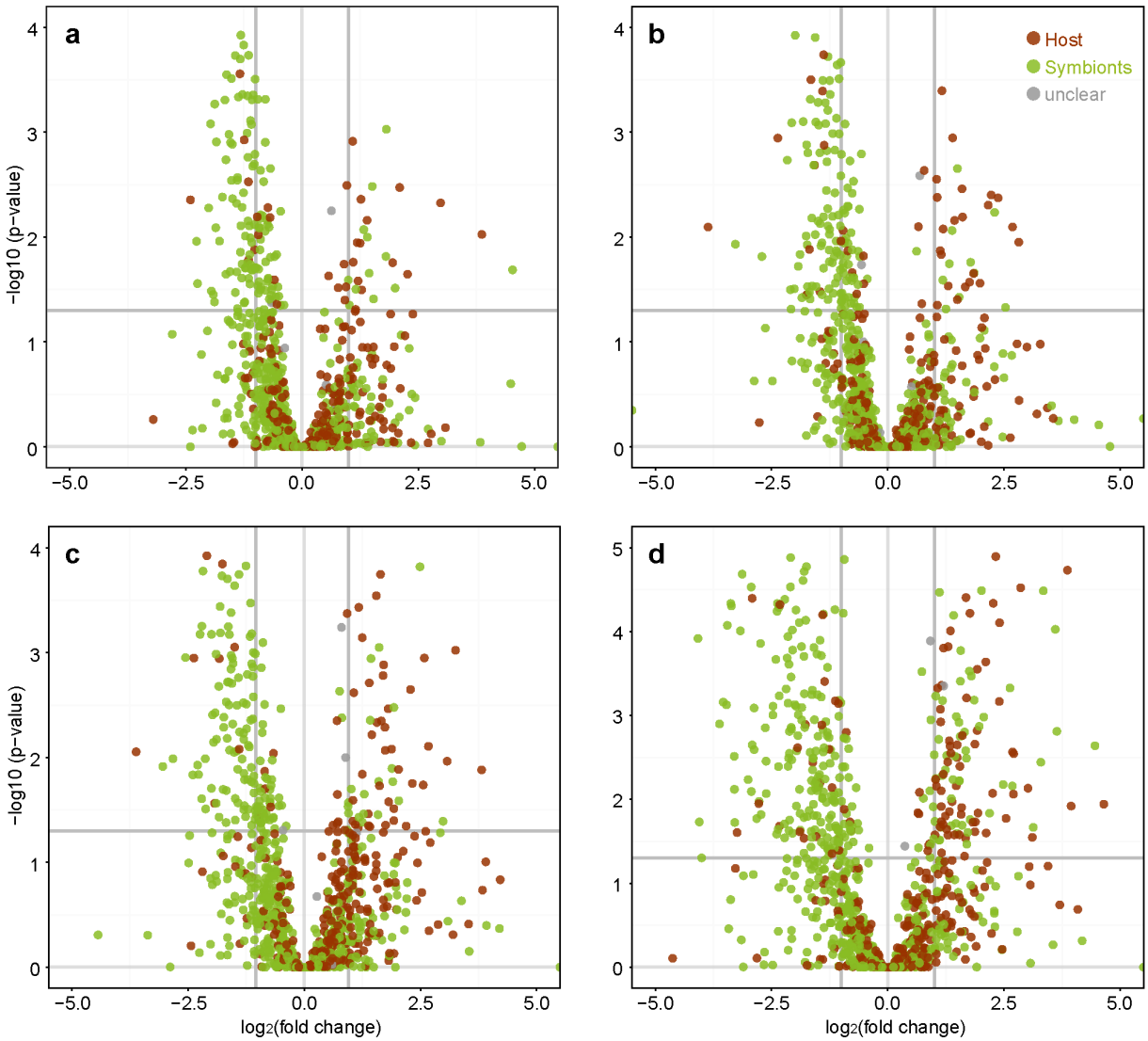

Figure S4-8. Volcano plots of log_2 fold changes versus –log10(p-values), representing the probability that the protein is significantly regulated, of all identified proteins in Amphistegina gibbosa *response to the thermal-stress treatments (a) control / no stress, (b) single stress event, (c) episodic stress events, (d) chronic stress (please note different scale of y-axis), compared to the start. Proteins above –log10(0.05) = 1.301 are considered significantly regulated where fold changes of above $–log_2(2) = 1$ specify increased and below $–log_2(0.5) = –1$ specify decreased abundances.*

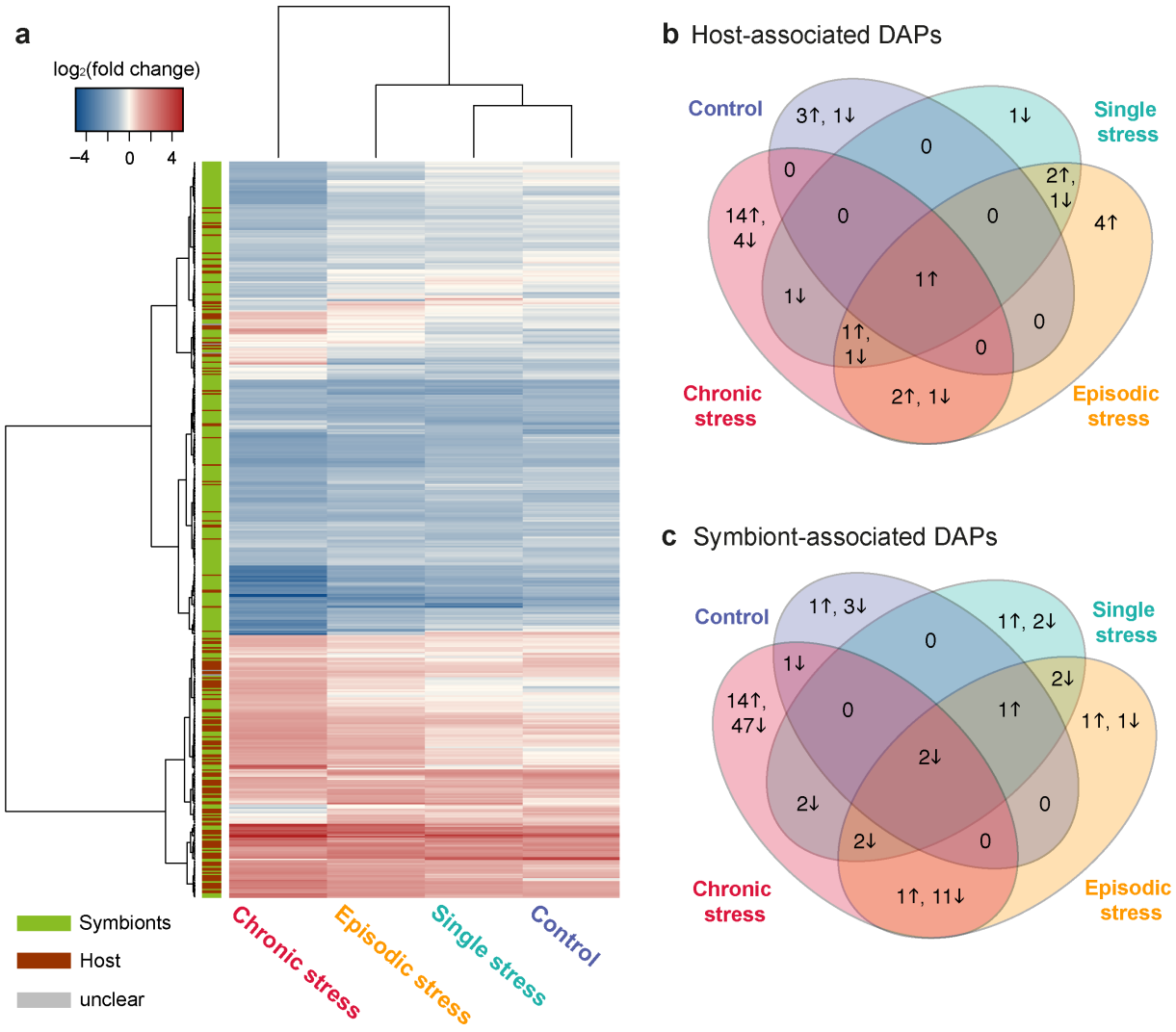

Figure S4-9. The heatmap and hierarchical cluster analysis (Euclidean distance) of all regulated proteins compared to the start (a) illustrates the direction of log_2 fold changes in protein abundances and their distribution among host foraminifera (brown) and symbionts (green). The normalized abundance values, significant p-values, and accessions of all regulated protein clusters are reported in Table S4-3. Venn diagrams of the amount of proteins that significantly changed in abundance in response to control conditions (blue), a single stress event (turquoise), episodic stress events (orange) and chronic stress (red) compared to the control in (b) the host (n = 37) and (c) the symbiont compartment (n = 92). Overlapping areas show protein groups that were equally regulated in more than one treatment. Arrows indicate how many proteins were up (↑) or down (↓) regulated.

Tables S3-4 and S4-4 represent Excel files that can be found in the data repository PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.883791) and will be made available online along the publication of the presented manuscript.

Chapter Five

EXTENDED DISCUSSION AND CONCLUSIONS

To evaluate and predict the future of an organism or ecosystem, we have to improve our understanding of causation between environmental history, acclimatization or adaptation and resilience. The overall objective of this thesis was thus to fill current knowledge gaps regarding the impact of ocean warming on photosymbiotic foraminifera and to disentangle characteristics of the host and the symbionts that influence their adaptive capacities. In order to gain a better understanding of future resilience of LBF under global environmental change, a range of physiological studies focusing on various organismal levels were conducted that jointly allow investigating the contributions of different mechanisms to adaptive responses in the holobionts.

To appropriately address this goal, three research questions were addressed:

i) Are there differences in LBF thermal stress response between species or within one species living in different habitats, and how do these correspond to associated symbiont species?

ii) Do temperature fluctuations in the form of single or episodic thermal peaks affect LBF the same way as chronic thermal stress, or do they induce acclimatization?

iii) What are the underlying cellular mechanisms of thermal stress responses in the host foraminifera and their symbionts?

The studies within the current thesis emphasize the complexity of interactions between foraminifera and their photosymbionts, as well as their environment. LBF have persisted in the worlds' oceans through vast geological time scales (~350 million years) (Hohenegger 2011) and the fossil record contains a rich proof of photosymbiosis in a variety of taxa such as the giant *Nummulites* (Stanley Jr and Lipps 2011). The various forms of LBF represent adaptions to different living conditions, optimizing the energetic benefit gained from the symbiosis under a given climatic setting (Lee and Hallock 1987). The driving evolutionary force of this photosymbiosis is already illustrating its great importance for LBF prospering. Latest studies have shown a multitude of negative impacts connected to the disruption of photosymbiosis by environmental stressors (Toler and Hallock 1998; Williams and Hallock 2004; Hallock et al. 2006; Schmidt et al. 2011; Prazeres and Pandolfi 2016). As bursts of evolution in foraminifera have usually taken place during times of strong climate change (Lee and Hallock 1987), LBF seem to have developed certain characteristics that allow them to adapt fairly quick to new living conditions. These features may considerably influence their persistence and resilience towards

environmental changes they are exposed to today and will be in the near future (IPCC 2013; Hoegh-Guldberg et al. 2017).

The results of the experiments conducted within the frame of this thesis indicate that while all tested *Amphistegina* populations are generally able to cope with large thermal fluctuations, sustained warming leads to a disruption of the symbiotic relationship (chapters 2, 3 and 4). Additionally, the different levels of physiological responses, from proteins and cellular processes to the performance of the entire holobiont, gave novel insights into the underlying mechanisms and potential interactions between both symbiotic compartments (chapters 3 and 4). Trying to disentangle the possible causes of inter- and intra-species variations in thermal stress responses, such as differences in host and symbiont species adaptations or different environmental conditions in their local habitats that stimulate different thermal acclimatizations (chapter 2), further highlighted the complexity of such efforts.

The role of flexibility / specificity in the endosymbiosis with diatoms and the potential adaptive mechanisms in photopigment composition may play a central role in resilience mechanisms of LBF and require further exploration. Furthermore, while an experimental exposure time of one month is a relatively large proportion of the lifetime of foraminifera, it did not allow to confidently identify possible trade-offs of the effective stress responses. These, however, need to be considered and are discussed below. Along the lines of very recent advances in coral research (e.g., Bay and Palumbi 2017; Thomas and Palumbi 2017; Ziegler et al. 2017), the presented results demand for further investigations using refined experimental designs. Employing modern molecular tools in photosymbiotic holobiont research offers immense opportunities (Silvestre et al. 2012; Doo et al. 2014b; Mayfield et al. 2014, 2016b; Oakley et al. 2016; Weston et al. 2015) that can only be scratched upon here. Lastly, some recommendations are made for improvements of the utilized methods and potential future applications in LBF ecology and environmental monitoring.

5.1 The effects of environmental fluctuations

The foregoing chapters showed that *Amphistegina* species seem to have a comparatively high thermal tolerance in general, supporting former studies that found their threshold mostly approximating 32°C for long-term exposure (Talge and Hallock 2003; Schmidt et al. 2011; Engel et al. 2015; Prazeres and Pandolfi 2016; Prazeres et al. 2016b), even in environments where it doesn't appear to have any benefit (Schmidt et al. 2016a). The obtained results also show that the consequences of thermal stress depend highly on the persistence of the exposure temperature. The amount of proteome variations in the different thermal-stress treatments reflects this stress pattern, from minor regulations after a single stress

event to clear adjustments of cellular processes after episodic stress events, and major impacts on both symbiotic partners under chronic stress exposure.

Contrary to the expected induction of thermal stress by a three-day thermal peak up to 32°C, the single-stress treatment did not significantly affect any of the three populations (chapter 2). Since there was no remarkable impact, no assumptions on recovery can be made. However, specific reactions to single and episodic stress-event treatments are evident in all populations, and these are distinct amongst populations. For example, impacts on the photosymbiont performance were predominantly visible in *Amphistegina gibbosa* from 18 m depth if thermal peaks recurred episodically, indicated by lower Chl *a* concentrations, *b** values and photosynthetic rates. Monitoring physiological parameters over different time steps revealed that the divergence of the episodic-stress treatment only developed between days 12 and 21, hence after the second or third peak (chapter 3). The same accounts for Chl *a* of the population of *A. gibbosa* from 5 m depth, whereas episodic-stress treatment did not significantly impact *A. lessonii* at any time (chapter 2). Hence, I conclude that all *Amphistegina* populations tested in this thesis are adapted to occasional pulsed heating events up to 32°C, but only *A. lessonii* from Zanzibar can cope with the repeated exposure to such conditions without requiring further adjustments.

The insights gained from proteomics in Ag18 reveal that, in contrast to chronic stress, single- and episodic-stress treatments mainly induce protein regulations in the symbionts (chapter 4). This can be due to a higher sensitivity to elevated temperatures of the endosymbiotic diatoms compared to the host foraminifera. Studies on other LBF found that in response to thermal peaks, net photosynthesis became negative and respiration rates decreased, but whilst the latter recovered within less than a day, recovery from photosystem damage took several days (Fujita et al. 2014). As all physiological parameters and the proteome responses were only analyzed after the thermal stress events were slowly abated over one day, the LBF already had time for recovery. These results thus do not infer the acute impacts of thermal peaks and we expect that proteome responses of the host would be higher if sampling was done during the peak. Nonetheless, the studies within this thesis suggest that the foraminiferal hosts are resilient to thermal fluctuations, while the endosymbiotic diatoms of *A. gibbosa* were marginally impacted by the thermal stress events. Due to the mild character of thermal peak impacts on symbiont parameters, no obvious acclimatization over the term of the experiment can be inferred. Despite an initial decrease of Chl *a* concentrations in Ag5 and Ag18, symbiont densities seemed to rise towards the end of the experiment. Such a pattern would indicate at least recovery.

Figure 5-1. Empty tests of Amphistegina gibbosa *from the control. The bright white color with small black spots are typical for specimens that reproduced sexually (P. Hallock, personal communication; scale bar: 0.4 mm).*

For the hosts, sexual reproduction (gametogenesis), seen as empty shells or mortality (chapter 2), was highest in the single-stress treatment in both *A. gibbosa* populations (Fig. 5-1). Reproduction only started to occur after the first or second stress event, and the sole reproductive event observed in the episodic-stress treatment occurred at the end of the experiment in the shallow-dwelling population Ag5. The single stress event may thus have advanced reproduction in *A. gibbosa*, while episodic-stress and chronic-stress treatments suppressed reproduction almost entirely. Similar reproductive failures have been described as a stress response in corals (summarized in Glynn 1996; Baker et al. 2008) and equally, the fecundity of *A. lobifera* was reduced by elevated temperature (Prazeres et al. 2017b). Moreover, host proteome data from Ag18 (chapter 4), in particular from the single and episodic-stress treatments, show elevated abundance of the actin-related protein 2 (arp2). It is part of the Arp2/3 complex that is responsible for actin filament nucleation (Mullins et al. 1998). Hence this protein is important for cell motility (Travis and Bowser 1986) and its up-regulation may be related to the trend of highest motility in this treatment. By climbing to elevated positions the LBF may accelerate the dispersal of the released gametes. Alternatively, the pre-reproductive cellular reorganization may require enhanced restructuring of actin filaments. While these postulations remain to be further examined, a suppression of reproduction would have far-reaching implications for LBF communities (Hallock et al. 1995) and ultimately affect reef carbonate budgets (Reymond et al. 2013; Doo et al. 2014a).

5.2 Indicators of oxidative stress

In response to chronic thermal stress, all populations showed the bleaching response previously described in several other LBF species (Talge and Hallock 1995; Schmidt et al. 2011, 2014; Doo et al. 2014a; Prazeres et al. 2016b; Waters and Hallock 2017). However, the exact causes and mechanisms of the loss of symbionts or pigments remained unresolved. Reactive oxygen species (ROS) are an inevitable by-product of aerobic metabolism. Their over-production, initially caused by damage to photosystems, has been commonly linked to the compromization of symbiont chloroplast thylakoid membranes and results in the subsequent breakdown of marine photosymbiosis, especially in response to thermal stress (Lesser 2006). In non-endosymbiotic diatoms, similar oxidative stress mechanisms

have been described in response to iron limitation, leading to programmed cell death (Luo et al. 2014).

Organisms usually protect themselves from the harmful effects of ROS by different enzymatic and non-enzymatic compounds that jointly signify complex and efficient antioxidant defenses (Lesser 2006). The steep increase of total antioxidant capacity against peroxyl radicals (ACAP) measured in the chronic-stress treatment of Ag18 (chapter 3) clearly confirms that sustained heat caused the requirement to enhance the ROS defense system. It was the first time that chronic thermal stress was shown to induce such constantly increasing ACAP in LBF over an extended timeframe. This confirms studies on effects of metal exposure that demonstrated that *A. lessonii* produce less ROS (i.e., have higher antioxidant capacities), and exhibit higher levels of lipid peroxidation, metallothionein-like protein concentration and total SOD activity when they are in the early stages of bleaching compared to normal appearing specimens (Prazeres et al. 2011, 2012). This further hints to an activation of antioxidant defenses in pre-stressed LBF, which consequently augments their resilience to subsequent stressors and highlights potential actors in ROS removal. Comparisons to *A. lobifera* populations from different habitats (Prazeres et al. 2016b) indicate that the ability of LBF to constantly increase ACAP or recover from an initial heat stress-induced reduction of ACAP is not universally given, but instead characterizes populations with elevated resilience. ACAP and proteomics analysis were only applied in Ag18 (chapters 3 and 4), the in comparison least thermo-tolerant population (chapter 2) tested in this thesis. Employing these methods to compare populations is highly recommendable for upcoming studies, as these should reveal different intensities of cellular responses and thereby demonstrate key pathways and antioxidant defense mechanisms that lead to different thermal tolerances.

With this in mind, it is remarkable that despite the high antioxidant capacities found in chapter 3, no antioxidant up-regulation was discovered in the proteomes of either compartment (chapter 4). While one peroxidase (phospholipid methyltransferase) decreased severely in the symbiont compartment, no common oxygen radical scavengers were adjusted in the host. Thermal stress in *A. gibbosa* induced proteomic changes that imply protein repair, degradation and re-synthesis, but no typically regulated antioxidants such as superoxide dismutase (SOD) or catalase were significantly elevated. Hence, either the proteins acting as antioxidants could not be identified as such by our confined database or the subsequent GO annotation, or several different scavengers were up-regulated simultaneously in a way that the total capacity increased considerably but none of the antioxidants was elevated outstandingly. Besides, those proteins that were increased and pertain multiple functions such as e.g., calreticulin, may fulfill detoxifying functions.

Similarly surprising observations were made in heat-shocked *Aiptasia* (Oakley et al. 2017), and coral hosts exposed to high temperature and light (Weston et al. 2015). As alternatives, uric acid, choloylglycine hydrolase, glutathione and tyrosine, which can be synthesized by the enzyme enolase 2 in the host, and many others compounds were suggested to play a role as ROS sinks in cnidarians (Meyer and Weis 2012; Oakley et al. 2016, 2017; Weston et al. 2015). In other marine animals and diatoms, the thioredoxin–peroxiredoxin system and other enzyme machineries also seem to play important roles, just like non-enzymatic feedbacks, i.e., switching from pathways that produce ROS (e.g., the tricarboxylic acid cycle and the electron transport system) to reactions that produce the reducing nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., the pentose phosphate pathway), acting as ROS scavenger (Luo et al. 2014; Tomanek 2015; Dong et al. 2016). Consequently, potentially important ROS scavengers in LBF were likely overlooked, highlighting that our understanding of functional proteomics needs to be improved (Vaudel et al. 2013). Additionally, it should also be considered that protein abundance is not synonymous to protein activity. A high amount of potential antioxidants might always be present in the cell, but their activity is only stimulated if required. Measuring total antioxidant capacities via the potential of ROS removal (as in chapter 3) integrates both, antioxidant abundance and activities, and therefore represents a valuable addition to quantitative proteomics (as in chapter 4) by giving an estimate of the effectiveness of the observed cellular responses.

5.3 Bleaching in photosymbiotic foraminifera

While the causes of bleaching under chronic thermal stress can without much doubt be attributed to the production of harmful ROS, their exact way of disrupting the symbiosis between foraminifera and diatoms remains to be further elucidated. It was formerly argued that in order to reduce stress of ROS produced by the symbionts, the host might actively eradicate and digest the diatoms, as seen by numerous lysosomes surrounding shrunken symbionts in specimens of *A. gibbosa* (Talge and Hallock 1995). In this case, it would be expected that the host produced higher amounts of digestive enzymes, autophagy-related and cell degrading proteins. The host proteome of partially bleached LBF however, only confirmed this theory to a very limited extent. Indeed, membrane-related component decreased and lyase activity proteins increased, but only associated to the symbionts (chapter 4). While this may be a bias of our homology-driven search approach that can only associate proteins to the host, which are present in the Rhizaria sequences in the concatenated database, the high proportion of cell-death related proteins that were associated to the symbiont compartment further supports the hypothesis outlined in chapter 4 of diatoms deterioration by programmed cell-death. Likewise, Lee et al. (2016) described symbionts in *Marginopora* undergoing cell death by apoptosis, with no lysosomes associated to or near the symbiosome membrane.

This stands in contrast to the study of Talge and Hallock (1995) who described that the progress in field-bleached specimens starts with deterioration of cellular and intracellular membranes, followed by digestion of symbionts by the host, and ends with disintegration of organelles and granulation of the host cytoplasm itself. A later study showed that both photic and thermal stress reduce numbers of viable symbionts in *A. gibbosa*, but degraded endoplasm was only seen in response to light stress (Talge and Hallock 2003). In response to high light levels, also the ACAP of *A. lobifera* was found to decrease significantly (Prazeres et al. 2016a), opposing the steep increase or recovery of ACAP in thermal stress experiments (chapter 3; Prazeres et al. 2016b). These results give further evidence that the host is more resistant to elevated temperatures than to high light (Talge and Hallock 2003) and hence suggests that the digestion of symbionts (Talge and Hallock 1995) may be specific to host distress induced by irradiance. In combination with the proteomic results (chapter 4), the observations of Lee et al. (2016) and studies focusing on photobiology of LBF symbionts under temperature stress (Schmidt et al. 2011, 2016a; Doo et al. 2012b; Uthicke and Fabricius 2012; Fujita et al. 2014; Prazeres et al. 2016b) suggest that sustained high temperatures severely damage the photosystems, which may ultimately result in symbiont cell death. In contrast, high temperatures impact the host less directly. The observed impacts of ocean warming on LBF holobionts such as reduced growth and fecundity (chapters 2 and 3; Schmidt et al. 2011, 2016a; Prazeres et al. 2017b) are certainly a result of loss of functional symbionts. Because these are the major source of nutrition in photosymbiont-bearing foraminifera (Kremer et al. 1980; Lee and Hallock 1987; Hallock 2000), their deterioration leads to host starvation, as also evident from the distinct changes in metabolism (chapter 4).

5.4 Adaptive mechanisms of holobiont resilience

Foraminifera are known for their high phenotypic plasticity, for example by changing test morphology in response to altered environmental influences (e.g., Hallock and Hansen 1978; Hallock et al. 1986; Toler and Hallock 1998), and even clonal cultures have been observed to develop very different morphotypes by experimentally modified conditions (Pawlowski 2000). The plasticity of foraminiferal tests constitutes a major challenge when distinguishing between morphospecies and ecophenotypes (Haynes 1992). DNA sequences that have emerged in recent years allow for distinguishing between the abundant cryptic species (Pawlowski 2000; Kucera and Darling 2002). However, because foraminiferal DNA has some unusual features, some genera remain inaccessible by these methods (Pawlowski and Lecroq 2010; Habura et al. 2011). For *Amphistegina* spp., first successful results have only been reported very recently (Schmidt et al. 2016a). We therefore did not employ genetic methods to distinguish between host genotypes. While we can be quite sure that *A. lessonii* from Zanzibar and *A. gibbosa* from the Florida Keys belong to genetically different species, we can only presume that both *A. gibbosa*

populations represent the same genotype due the close proximity between sampling sites. This assumption is strengthened by *A. lobifera* populations from the Red Sea and the Mediterranean that cannot be distinguished by DNA sequencing despite that they must have been separated over many generations and live in very distinct environments (Schmidt et al. 2016a). The high homogeneity of their symbiont assemblages and multivariate analysis of physiological responses to different thermal-stress scenarios (chapter 2) indicated high similarity. Intra-species variations were hence minimal and suggest that the shallower-dwelling populations may be acclimatized to marginally higher temperatures without an influence of different host or symbiont species.

5.4.1 The thermal tolerance of *A. lessonii*

In contrast to intra-species variances, the different *Amphistegina* spp. were clearly distinguishable from each other by their physiological responses, especially based on those parameters indicating symbiont performance (chapter 2). *A. lessonii* was found to have a generally higher thermal tolerance, seen by the absence of significant impacts of the episodic-stress treatment and less pronounced responses to chronic stress. This confirms former studies on these amphisteginids. Experiments comparing light preferences of these two species (Hallock et al. 1986; Walker et al. 2011) revealed that *A. lessonii* is adapted to higher levels of PAR. First of all, this means that light levels in the presented experiments, adjusted to the upper level of irradiance not causing damage in *A. gibbosa* (Talge and Hallock 2003; Williams and Hallock 2004), were probably below optimum for Al5. LBF grown at reduced light conditions decrease their antioxidant capacities and shell density, but not their surface area-based growth rates (Hallock and Hansen 1978; Prazeres et al. 2016a). Hence, under optimum light levels, Al5 would possibly have performed even better, but the interactions between low light conditions in combination with thermal stress remain a topic for future research. Secondly, higher light preferences indicate that their symbionts might be generally less sensitive, reducing their potential to induce oxidative stress by photosystem malfunctioning.

Exposure of seven LBF species to a high temperature and low pH environment in the proximity of shallow-water hydrothermal vents highlighted the low sensitivity of *A. lessonii* compared to *A. lobifera* and *A. radiata* originating from the same sampling site (Engel et al. 2015). These researchers even reported specimens 'living in algal-microbial films exposed directly to vent fluids on volcanic rocks that were noticeably hot to the touch' (Engel et al. 2015, p. 199). Moreover, *Amphistegina* spp. that appear to be – at least partially – *A. lessonii* (Weinmann and Langer 2017, plate 2, 7a-b) were present in tide pools often exceeding 35°C. It is thus feasible that *A. lessonii* is resilient to thermal and other stressors and notably may have the potential to thrive in extreme habitats. Such higher threshold can have multiple reasons (Weis 2010) such as acclimatization, genetic adaptations of the host or the

endosymbiotic diatoms, or higher flexibility in the associations with various symbiont species that allows rapid adjustments. In corals, all these adaptive mechanisms have been reported as pivotal factors for enhanced thermal tolerance of the holobiont (Baker 2003; Barshis et al. 2010; Howells et al. 2011; Oliver and Palumbi 2011a; Mayfield et al. 2012, 2013; Palumbi et al. 2014). Testing the genetic basis of thermal tolerance requires controlled experimental conditions and represents interesting potential for futures studies. Nevertheless, some assumptions on the influence of possible acclimatizations and symbiont assemblages on the different thermal tolerances observed in chapter 2 can be made.

5.4.2 Thermal conditions of the environment

Acclimatization would result from prior stress events. Although *A. lessonii* has been cultured at control temperatures for several (~10) weeks before the start of the experiment, the population might have been already acclimatized to thermal peaks above 30°C and retained this tolerance. The LBF were sampled in April, shortly after the warmest period of the year in Zanzibar, Tanzania, which usually occurs by the end of March (Muhando 2002). No thermal records for Changuu Island are found that could provide insights into the water temperature fluctuations prior to sampling. But based on model-derived air temperature data (e.g., windguru.org) and observations from other years (Muhando 2002), it seems likely that seawater temperatures in shallow areas temporarily reached above the measured mean temperature of 29°C, though prolonged temperatures of ≥ 32°C are doubtful.

Recent stress events in the thermal history of corals can enhance photoprotective mechanisms and thereby lower symbiont loss until at least 2 weeks later (Middlebrook et al. 2008). How long such 'immunization' lasts is not known, but recent studies have revealed that the transcriptome of coral hosts remained largely perturbed for at least half a year after a severe disturbance and did not fully recover even after one year (Thomas and Palumbi 2017). While the investigated natural coral bleaching event was devastating compared to the simulated single stress event in this thesis, multivariate analysis of proteome responses in Ag18 clearly displayed impacts until 27 days after the end of the event (chapter 4). Jointly, these retained impacts on the LBF proteome and coral transcriptome (Thomas and Palumbi 2017) suggest that exposures to high temperatures during summer maxima may induce long-lasting cellular acclimatizations to cope with thermal stress, remaining active for several months.

5.4.3 Influence of the symbiont assemblage

All identified diatoms in this thesis belong to the order of Fragilariales (chapter 2). Those within *A. gibbosa* are dominated by a single type that has not been described before as free-living, but is closely related to *Opephora* and *Staurosira* spp., uncultured eukaryotes (diatoms) extracted from *A. lobifera* (Schmidt et al.

2016a), and an unnamed Fragilariaceae sp. cultured from sand grains in the Florida Keys (Barnes 2016). The endosymbionts in *A. lessonii* were also closely related to these, but much higher diversity of types was found. Diatoms are a very diverse group comprising an estimated 200,000 species (Mann and Droop 1996), but only few model-species have been thoroughly assessed regarding their ecological preferences (Muhseen et al. 2015). Particularly the pennate diatom genera often described as foraminiferal endosymbionts (Lee and Correira 2005; Holzmann et al. 2006; Lee 2006; Schmidt et al. 2015, 2016; Prazeres et al. 2017a, chapter 3) that predominantly belong to the smallest known clades of diatoms (Fig. 5-2), have been poorly described in general (Li et al. 2016). It is thus impossible to make assumptions on the ecology and thermal threshold of the diatom taxa detected here, highlighting the need for advance research in this field.

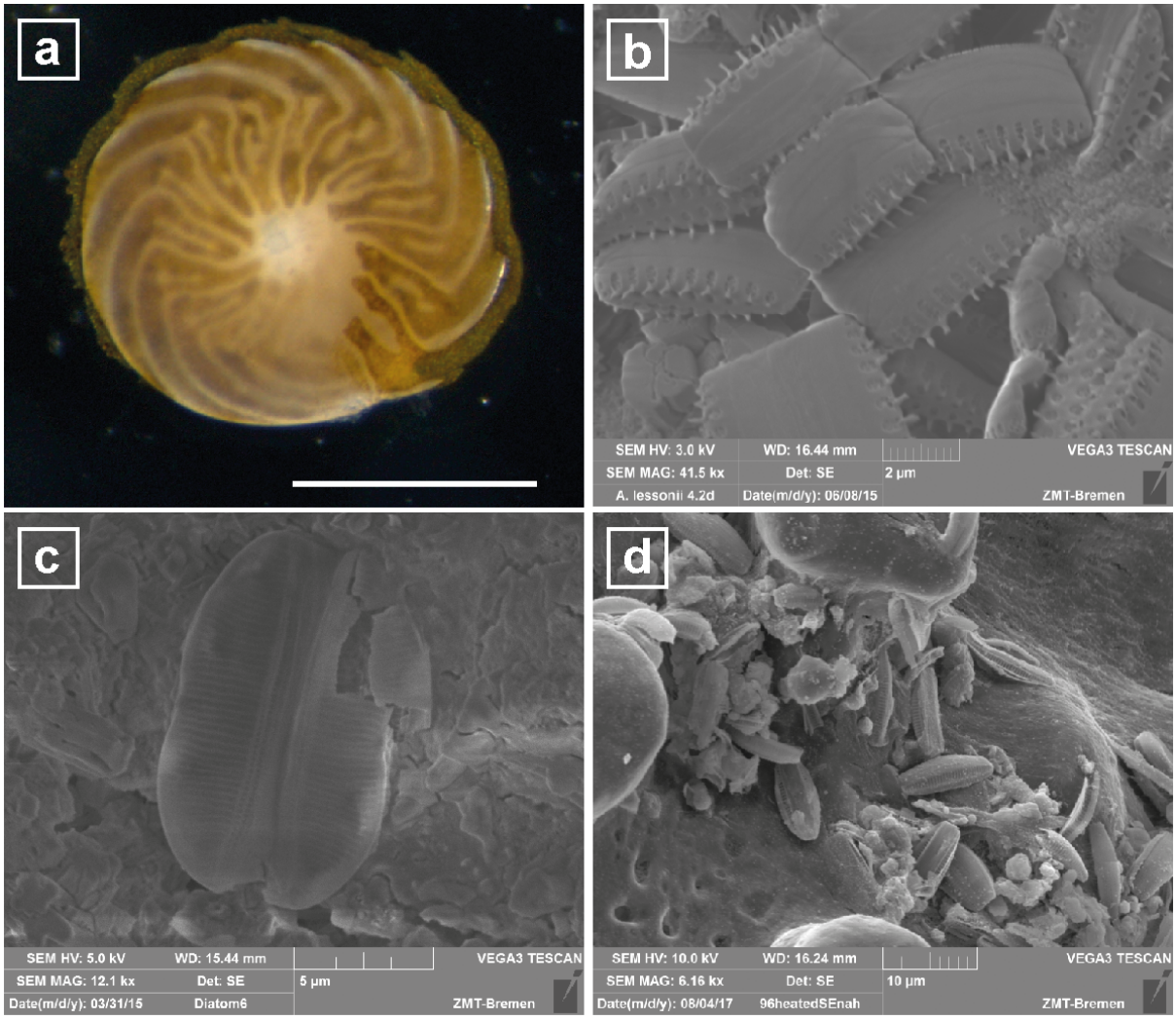

Figure 5-2. Diatoms associated to Amphistegina*: a) dissolving the calcareous test releases the endosymbionts so they may be extracted and cultured, scale bar: 0.5 mm; b) and c) diatoms found in cultures of* A. lessonii *extracts; d) diatoms found on the aperture of* A. lobifera *that might serve as food or potentially for symbiont re-assimilation.*

Another possible reason for more resilient symbiotic associations is a higher flexibility that allows stress-induced symbiont shuffling as observed among cnidarians (Baker 2003). It is not entirely clear whether LBF can re-assimilate symbionts from their environment after severe bleaching (Lee et al. 1986). But personal observations (M. Stuhr) of 'recoloration' of partly bleached specimens isolated in petri dishes (Fig. 5-3) propose that at least the remaining symbionts are able to replenish the host. This could likewise shift symbiont compositions, given that multiple lineages were initially present. Observations of biogeographic distinctions in dinoflagellate-symbionts in LBF (Pochon et al. 2004; Momigliano and Uthicke 2013), and regional differences in associations of diatom-symbionts (Holzmann et al. 2006; Prazeres et al. 2017a), suggest in any case a shaping influence of environmental conditions. Specificity and flexibility in photosymbiont-assemblages are hence likely to have a significant influence on evolutionary traits (Lee et al. 2010) and prone have bent the adaptive range of *A. lessonii*.

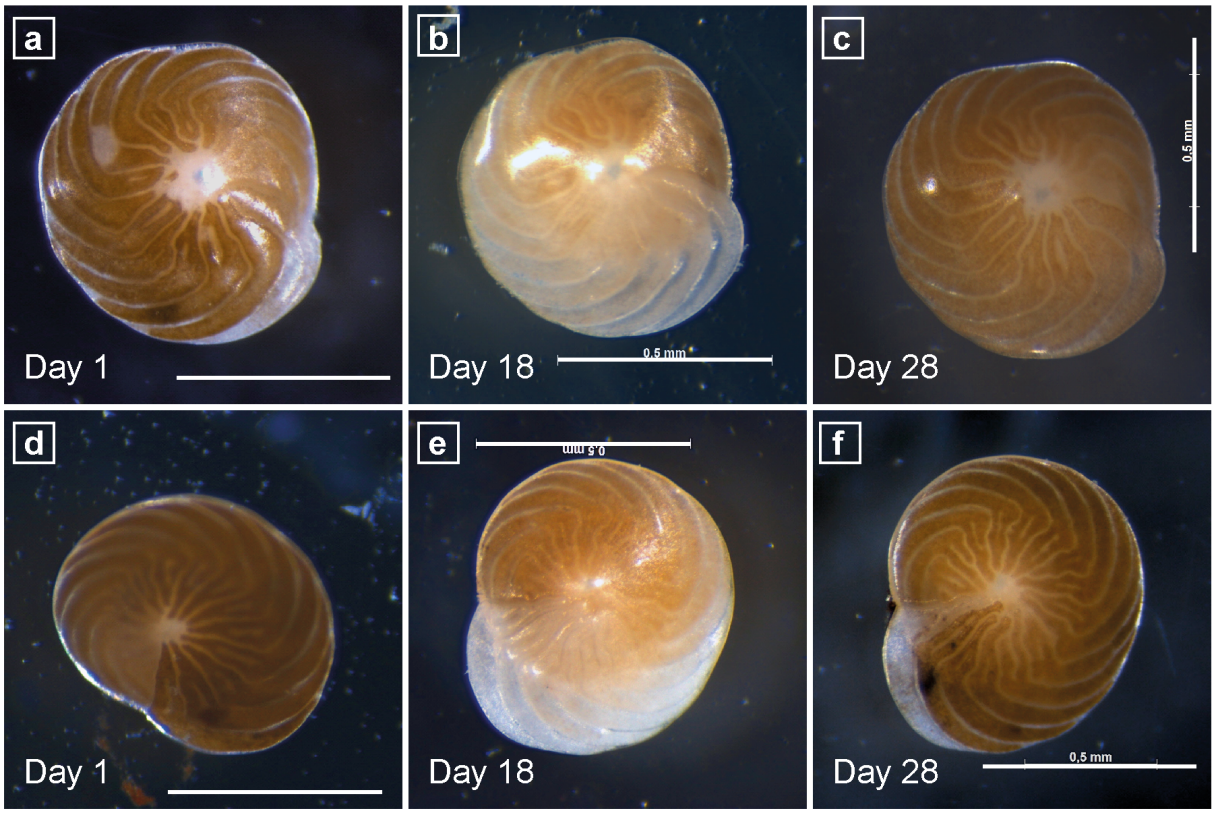

Figure 5-3. Recoloration of partially bleached Amphistegina *specimens after exposure to diuron: a-c)* A. gibbosa, *d-f)* A. lessonii. *Symbiont loss was induced by addition of the herbicide from day 1 (a, d) until days 18 (b, e). Afterwards, the water was replaced by herbicide-free water. By day 28 (c, f) coloration was widely re-established. Note also the disappearing of the local symbiont loss (a, white spot) in* A. gibbosa *and the black accumulation appearing in* A. lessonii *(f, scale bars: 0.5 mm).*

5.5 Conclusive remarks and implications

In summary, this thesis provides evidence that the responses of the LBF *Amphistegina* spp. to ocean warming largely depend on the persistence of thermal stress, but are also determined by host and/or symbiont species-specific factors and further shaped by their environment. All studied populations were generally resilient to single and episodic heating events, but showed severe impacts under chronic exposure to 32°C. The observed bleaching and related impacts on photosymbiont physiology (e.g., Chl *a* concentrations and photosynthetic rates) came along with reduced holobiont growth, and in *A. gibbosa* also reduced motility and constrained reproduction, confirming former thermal stress experiments on LBF (Talge and Hallock 2003; Schmidt et al. 2011, 2016a, Prazeres et al. 2016b, 2017b). However, all specimens survived the entire experimental period of one month and thereby showed a higher tolerance to sustained warming than many other species tested so far (Sinutok et al. 2011; Uthicke et al. 2011; Prazeres et al. 2017b).

Facilitated by a bottom-up proteomics workflow that was adapted for minuscule foraminifera samples, containing the carbonate tests and both symbiotic compartments, host- and symbiont-associated proteins were separated *in silico*, significantly regulated proteins were identified and insights into their molecular functions were gained (chapter 4). Multivariate analyses of their quantitative variations showed patterns that were highly congruent to the responses indicated by traditional physiological methods (chapter 3). This validated the presented novel approach and furthermore pinpointed the underlying cellular changes involved in the different thermal stress responses. These include disruption of carbon concentrating mechanisms, induction of cell death and degradation of the symbionts under chronic stress, as well as repair mechanisms by unfolded protein response and enhanced protein synthesis that maintained host homeostasis. Changes in metabolic pathways and intensification of microtubule-based processes indicate an intensification of heterotrophic feeding by the foraminifera to compensate for the loss of symbiont-provided photosynthate (chapter 4). Hence, reactions and adjustments of both compartments were revealed that were previously undetected. Because this methodology can be easily transferred to other photosymbiotic organisms, it offers numerous possibilities to study the ecology and adaptive mechanisms of coral reef calcifiers.

The pronounced responses to chronic stress, and the slight adjustments to single- and episodic-stress treatments exhibited clear species-specific differences. *A. lessonii* showed a higher thermal tolerance, which may be related to the different and more diverse diatom community detected (chapter 2), but could also be a genetic adaptation of the host or retained acclimatization to thermal peaks prior to sampling. Additionally, small intra-species differences between two populations of *A. gibbosa* originating from different water depths indicate a minor shaping

influence of local habitat conditions. As symbionts were highly homogeneous within this species, the slightly higher sensitivity of the deeper-dwelling population of *A. gibbosa* to thermal peaks is likely caused by lower mean temperature in their natural environment.

In light of the effects of climate change on ocean conditions, the results of this thesis indicate that at least some LBF species have an advantage over other marine calcifiers. *A. lessonii* seems to have a generally high threshold to thermal stress compared to other *Amphistegina* species, suggesting that their relative importance in future reef habitats will increase in the future. While the exact reasons for the elevated tolerance remain to be specified in future research, the character and flexibility of their associated diatoms is likely to play a key role. *A. gibbosa* in contrast might be limited in their adaptive capacity to thermal stress by their quite explicit symbiont-association. Nevertheless, the resistance of *A. gibbosa* to thermal fluctuations indicates that organisms that are naturally exposed to such heterogeneous environmental conditions can adapt to transient heating events (chapter 3). Organisms from highly variable habitats such as reefs influenced by large-amplitude internal waves (Roder et al. 2011; Buerger et al. 2015) and temporary upwelling (Mayfield et al. 2012, 2013), intertidal or rock pools (Oliver and Palumbi 2011b; Weinmann and Langer 2017) can therefore represent important source populations for restocking. They may also harbor a genetic basis for thermal tolerance that can potentially facilitate rapid adaptive responses (Torda et al. 2017). Global conservation efforts should therefore focus on marine habitats with fluctuating thermal regimes, as well as species of generally elevated resilience.

The presented inter- and intra-species variations, and the distinctions between physiological responses to temperature fluctuations and sustained warming have further implications for the application of LBF as bioindicators. In fossil and current assemblages, variable adaptation and acclimatization due to environmental setting should be taken into account as far as possible, and for some species or locations the thermal ranges may be higher than formerly assumed. Although amphisteginids represent sensitive indicators for photic stress, heat-induced bleaching in the tested populations only occurs at levels and durations of high temperature at which other photosymbiotic reef organisms are already impacted. If tools that give an estimate of the physiological cellular state, such as shotgun-proteomics or biomarker assays, are included into monitoring programs, they could provide highly sensitive indicators of recent environmental history and stress condition.

The recognized thermal tolerances and adaptive capacities of the examined diatom-bearing foraminifera may advance their resilience towards ocean warming. However, an increase of mean seawater temperature of ~3°C by the end of the century, as expected under the business-as-usual emission scenario RCP8.5 (IPCC

2013), would still severely impact *Amphistegina* spp. in shallow realms, especially during summer months. In the long-term, the impairment of reproduction and growth would not only reduce LBF communities, but also diminish their contribution to calcium carbonate sediment production (Hallock et al. 1995; Reymond et al. 2013; Doo et al. 2014a). Ultimately, this could impair their function for replenishing reef-islands, stabilizing sand cays and coral reef habitats (Hohenegger et al. 1999; Yamano et al. 2000; Dawson and Smithers 2014), and locally buffering seawater acidification by post-mortem test dissolution (Yamamoto et al. 2012). Furthermore, the symbiosis with photosynthesizing microalgae is not restricted to LBF, but also widespread among planktonic species. Since the disruption of this symbiosis under thermal stress seems to be mostly due to the deterioration of photosymbionts, similar mechanisms may act in planktonic conspecifics that are of even higher importance to global geochemical cycles (Langer 2008). Indeed, the fossil record indicates that former planktonic foraminiferal extinctions were preceded by the loss of photosymbiosis (Wade et al. 2008).

Besides thermal stress, foraminifera are impacted by a multitude of other global and local stressors such as ocean acidification, pollution, eutrophication and intense irradiation (Hallock et al. 1995; Reymond et al. 2011, 2013; Uthicke and Fabricius 2012; van Dam et al. 2012; Ross and Hallock 2014; Marques et al. 2017). Earlier studies have highlighted the mostly additive or synergistic effects of combined stress factors (Talge and Hallock 2003; Uthicke et al. 2011; Schmidt et al. 2014; Marques et al. 2017; Prazeres et al. 2017b), which implies that resilience to thermal stress alone is not sufficient to withstand future climate change if other stressors co-occur.

The results of the presented thesis contribute to an emerging body of studies that start to disentangle the underlying drivers of photosymbiotic reef organisms' responses to climate change, the interactions between host and symbionts, and factors influencing their adaptive capacities. Moreover, the applied dual-compartment proteomics approach provides a valuable tool for coral reef research, especially with respect to the quickly developing genomic datasets for numerous key taxa.

Chapter Six
OUTLOOK

6.1 Disruption of photosymbiosis and the role of communication

While the results of this thesis give detailed insights into compartment-specific changes in heat stressed and bleached LBF, open questions remain. A formerly suggested cause for bleaching in *Amphistegina* spp. is damage to the CSSA (Common Symbiont Surface Antigen) glycoprotein, which is found on symbiont surfaces to protect them from digestion (Lee 2006). Our results showed that symbionts are deteriorating independently of host digestion, but as mentioned before, this might be stressor-dependent or due to database-related biases. Prazeres et al. (2012) argued that injury of this protein would disrupt its protective function that keeps the symbiosis intact, leading to symbiont digestion (Talge and Hallock 2003). Furthermore, experiments with extracted diatom symbionts that were grown with and without host homogenates signify differences in frustule development as well as photosynthate release (Lee et al. 1984). This highlights the essential role of some shared actors to keep the symbiosis intact.

Studies on dinoflagellate-bearing LBF detected temporary re-location of symbionts in order to protect them from light stress, actively performed by the host (Petrou et al. 2017). Such rapid actin-mediated movement of symbionts within the host cell, and the phototactic movement of the whole holobiont to optimize light exposure (Zmiri et al. 1974) must consequently be induced by infochemical signaling between both compartments (Petrou et al. 2017). Likewise, the proteomics results of thermally stressed LBF and diatoms (chapter 4) showed that some of the proteins undergoing strongest increases in abundance were members of the small GTPase superfamily. Also enzymes involved in vesicle-mediated transport processes were up-regulated under chronic stress. These may be of high importance as mediators of cellular signaling during stress. Recent TEM studies captured previously unrecognized features such as vesiculated accumulation products and continuous oil droplets from the symbiont into the foraminiferal protoplasm (Lee et al. 2016), which support early studies that indicate that dinoflagellate-symbionts release lipid compounds rather than carbohydrates (Kremer et al. 1980). The latter study also showed that diatom-symbionts release relatively more glucose and glycerol than their zooxanthellate counterparts, but until today the exact trophic connections and molecular exchanges in LBF photosymbiosis are insufficiently understood (Lee 2006; Lee et al. 2016). Jointly, these observations emphasize the importance of communication for intimate host-symbiont interactions, and their role for resilience mechanisms therefore deserves more attention.

6.2 Image-based color measurements and photopigments

The *L*a*b** color value-based estimation of Chl a concentrations developed for the diatom-bearing *Baculogypsina sphaerulata* (Hosono et al. 2012) is not directly applicable to *Amphistegina* spp., because holobiont coloration is generally different. Likewise, a systematic difference in colors between *A. gibbosa* and *A.*

lessonii was noted. Not only with respect to measured Chl *a* values, but also considering which values were affected by the different thermal-stress treatments, it appears that bleaching and pigment loss do not respond uniformly among LBF species. This may be due to differences in test structure, or could result from variations in photopigment composition between foraminifera and/or symbiont species.

Pigment profiles can be resolved by high and ultra performance liquid chromatography (HPLC/UPLC) studies. Investigations detected remarkably low ratios of Chl a to chlorophylls c_1+c_2 and fucoxanthin (Fx) in *A. lessonii*, as well as high ratios of diadinoxanthin (Ddx) to diatoxanthin (Knight and Mantoura 1985). The authors attributed the relative lack of Chl *a* to stress during handling although this was not represented by an expected increase in degradation products. In *A. radiata*, the relative contributions of Fx, Chl c_2+c_2 and Ddx were not as high (Table 6-1), but still substantial (van Dam et al. 2012). In marine diatoms, high light stress can cause rapid photoacclimation including shifts in photopigment compositions, such as higher Ddx contents or a conversion of retinol to retinal (Dong et al. 2016). Altered light spectra or temperatures can likewise lead to fast photoacclimation (Kuczynska et al. 2015). Species-specific responses in colorations could thus be due to distinctive photopigment compositions and photoacclimations.

Table 6-1. Ratios of the pigments (X) chlorophylls c_1 *and* c_2 *(Chl* c_1+c_2*), fucoxanthin (Fx), diadinoxanthin (Ddx) and β-carotene (β-C) with respect to Chl* a *estimated in two* Amphistegina *species.*

Pigment ratios (X / Chl *a*)	**Chl c_1+c_2**	**Fx**	**Ddx**	**β-C**
A. radiata (van Dam et al. 2012)	0.48	1.34	0.12	0.04
A. lessonii (Knight and Mantoura 1985)	0.70	3.65	0.25	0.06

In this thesis, Chl *a* was measured via spectrophotometry (chapters 2 and 3) because 90% of its variation detected by UPLC can be predicted by this method (Schmidt et al. 2011). However, changes in other light-harvesting or photoprotective compounds cannot be detected by this approach. As there are no studies to date that directly compare the pigment composition in different *Amphistegina* species, nor the influence of environmental change on pigmentation in any LBF, the contribution of such mechanisms to local adaptations is a ripe target for future research. Such studies should be accompanied by species-specific calibrations of color values to photopigment concentrations. Besides, fluorescence-based imaging technologies that can excite and detect specific wavelength may be of additional value for distinguishing variations in fluorescent pigments such as chlorophylls (Fig. 6-1a). If reliable linear relationships between colors and environmental parameters can be found, then LBF could potentially be used as an even more sensitive indicator for these environmental parameters because slight

nuances in pigment loss or variation that cannot be detected by the eye can be detected by image-based analysis.

Figure 6-1. Severely bleached Amphistegina*: a) the exact areas of thermal stress induced bleaching can be visualized using fluorescence microscopy. The auto-fluorescent chlorophyll appears red, while cytoskeleton without functional symbionts appears green (credit: E. Freier, ISAS); b) upon exposure to the herbicide diuron, dark materials appear within some chambers that might work as protection; c) in response to high light intensities, some specimens accumulate red-brown materials; scale bars: 0.5 mm.*

Furthermore, opaque dark or red-brown pigments (Figs. 5-3f, 6-1b and c) can occur in response to stress (Hallock et al. 1995; Talge and Hallock 2003; Prazeres et al. 2011; Schmidt et al. 2011; Ross and Hallock 2014). These have been suggested to be moved between the outer test wall and the symbionts (e.g., Hallock & Hansen 1978; Williams & Hallock 2004) to serve as photoprotective compounds like microsporine-like amino acids (MAA), which are found in many other marine organisms (Sinha et al. 2000; Shick 2004). To date, these materials have not been characterized. They could potentially also be identified as degradation products of photopigments, e.g., phaeophytin, using HPLC techniques, or may instead be extracted and resolved by proteomics methods. Alternatively, time resolved sampling for proteomics analysis during ongoing bleaching or darkening of LBF could show which protein groups increase or decrease substantially in correlation with the changes in coloration, and thereby identify the compounds that hypothetically act in thermal and/or photoacclimation.

6.3 Improving and applying proteome analysis

The largest limitation to proteomic, but equally transcriptomic and metabolomics studies, is the availability of reliable databases, especially when working with marine non-model organisms (Tomanek 2014). Hence, these approaches are only able to detect and assign those proteins that are present in the used reference database. Based on the character of the sequenced organisms some genes/proteins might not be present, while others are highly abundant. Moreover, while some pathways have been investigated thoroughly (e.g., photosynthesis of terrestrial plants), others remain unresolved and so do the involved proteins. The genetic data of

foraminifera for example is broadly comprised of cytoskeletal proteins, owing partly to the fact that the first genome and EST libraries for this group (Burki et al. 2006; Glöckner et al. 2014) originate from the test-less freshwater species *Reticulomyxa filosa*, which comprises of a highly flexible cytoplasm. Another partial genome stems from the Antarctic cold-water foraminifera *Astrammina rara* that builds agglutinated tests of surrounding particles (Habura et al. 2011). Only relatively few ESTs were derived from a taxa building calcareous tests (Burki et al. 2007). Therefore, especially proteins required for calcification are likely to be underrepresented in our database and hence prohibit detecting their regulations.

Toler and Hallock (1998) noted a correlation of broken shells and malformations (Fig. 6-2) to cytoplasmic damage, hypothesizing that stressed individuals may have problems to produce enough glycosaminoglycans which could reduce structural integrity of their chambers. Furthermore, a limited serine- and glycine-rich protein synthesis may reduce the tensile strength of the test, whereas a disrupted or diminished production of highly acidic glycoproteins could disturb biomineralization (Toler and Hallock 1998). As other serine-interacting proteins were clearly decreased in the host (chapter4), depletion of serine synthesis may indeed be a factor that explains the impact of oxidative stress on test construction. Moreover, the strongest depleted protein in the host resembles an enzyme responsible for the synthesis of glycerophospholipids, the main components of biological membranes. Elucidating the role of these cellular compounds by genomic or proteomic tools may not only give deeper insights into stress responses, but also serve to investigate the still uncertain mechanisms of calcification (Bentov et al. 2009; de Nooijer et al. 2014) in foraminifera. However, this could profit immensely from an improvement of the reference database. If specific pathways are aimed for, future research should extend the assembled database by including more distantly related organisms or selectively incorporate sequences of proteins that are potentially fulfilling crucial functions in these pathways, i.e., research targeting calcification should cover enzymes that are knowingly involved in biomineralization. Ideally, sequencing and annotating the genomes of the studied organisms (here e.g., *Amphistegina* or a close relative), should accompany proteome analysis.

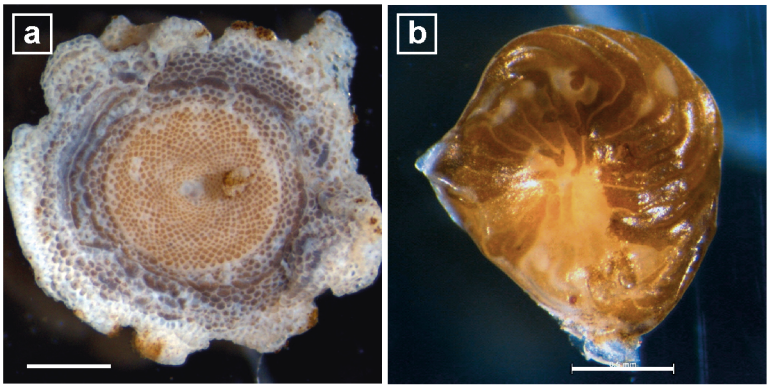

Figure 6-2. Photosymbiotic foraminifera from cultures, exhibiting deformed tests: a) Amphisorus *sp. (scale bars: 0.5 mm), and b) partly bleached* Amphistegina gibbosa *(scale bar: 0.3 mm).*

6.4 Proteomics and global change research

Despite the database-related limitations outlined above, a new view and better understanding of the specific functions and mechanisms involved in photosymbiosis can be provided by the comprehensive perspective of proteome analysis. By regarding the entire cellular system, possible trade-offs, but also to this point unrecognized adaptive processes such as the role of small GTPases in regulating vesicle transport in response to environmental stress could be recognized (Tomanek 2014). The large volume of gained information in relation to handling time of many samples simultaneously offered by gel-free proteomics approaches, and especially the very low amount of material necessary makes these techniques ideal for LBF (chapter 4) but also other photosymbiotic organisms such as cnidarians (Oakley et al. 2016). This even refers to organisms with low database coverage, but clearly, the amount and precision of protein identification and respective GO annotations is largely predetermined by the availability and quality of genomic or proteomic data (Vaudel et al. 2013).

Owing to the efforts of the coral research community to sequence multiple host and symbiont genomes (Shinzato et al. 2011; Baumgarten et al. 2015; Aranda et al. 2016; Tisthammer et al. 2016) in the last decade, the knowledge on the molecular ecology of the coral-*Symbiodinium* association is far ahead of the respective knowledge of LBF. To this adds that coral and anemone tissue can be easily removed from the calcareous skeleton and subsequently the dinoflagellate-symbionts and the host cells can be physically separated by centrifugation. Therefore, cnidarian photosymbiotic associations may be used to further assess the presented approach of *in silico* protein separation by comparing it to the compartment-specific results obtained when host and symbionts are separated *in situ* prior to protein digestion and MS-based peptide analysis. This would considerably strengthen the validity of the dual-compartment protein characterization and promote the transfer of the approach to the diverse other photosymbiotic associations in marine ecosystems, preferably those with high genome or proteome sequence coverage.

The bottom-up approach employed in this thesis is efficient in highlighting changes in relative abundance of all universally identified proteins. Thereby, it builds a basis for further analyses focusing on specific questions. For example, absolute qualitative changes in the abundance of certain proteins that are expected to play a critical role for the process in question can be targeted with isobaric tags such as iTRAQ (e.g., in Luo et al. 2014; Dong et al. 2016). Furthermore, post-translational modifications and protein-protein interactions are involved in the translation from signaling events of environmental impacts into biochemical responses at the cellular level (Tomanek 2014). Such adaptive responses that can act even independent of the transcriptome are only elucidated by proteomics approaches. With respect to

chapter 2, it would be highly interesting to obtain proteomic data from all investigated LBF populations and treatments. Intra- and inter-specifies variations in protein regulations that are key to understand the underlying differences in thermal stress tolerance could be elucidated this way, and at the same time the responsible symbiotic compartment would be identified.

To date, the transcriptome and gene expression are extensively studied to resolve adaptive traits in corals (DeSalvo et al. 2010; Mayfield et al. 2014; Moya et al. 2015; Bay and Palumbi 2017; Ruiz-Jones and Palumbi 2017), and occasionally also gel-based proteomics approaches are employed (Weston et al. 2015; Mayfield et al. 2016b; Ricaurte et al. 2016) that usually focus on one of the two symbiotic compartments. However, recent experiments have shown that coral transcriptomic variations hardly correspond to proteomic responses and hence the cellular phenotype (Mayfield et al. 2016b). Future research should therefore focus on the new perspectives given by proteomics. Especially the combination of shotgun (bottom-up) discovery proteomics, followed by targeted proteomics is vital for perceiving functional changes and hence develop biomarkers (Vaudel et al. 2013). Generally, omics methods are among the most powerful tools for investigating the mode of action of environmental stressors, and the detection of pathways involved in the establishment and maintenance of symbiotic relationships (Tomanek 2011; Meyer and Weis 2012; Voolstra et al. 2015). Combined with classical approaches, omics approaches and in particular proteomics, are essential to resolve persistent questions on the future of marine organisms (Tomanek 2014) and should therefore be intensified.

6.5 Tracing adaptive mechanisms in photosymbiotic organisms

An intact photosymbiosis is of central importance for the proliferation of many calcifying marine organisms, but the balance within this relationship can react delicately to changes in environmental conditions. Therefore, future studies focusing on the susceptibility/resilience of holobionts need to consider adaptation and acclimatization characteristics that contribute to stress response of both symbionts and hosts (Weis 2010). In order to gain insights into the adaptive mechanisms of LBF, elucidating the influence of specificity and flexibility in associations between symbiont and host species that have been recognized since more than three decades (Hallock et al. 1986; Lee and Hallock 1987) should be of high priority. This requires large-scale sequencing of both partners along environmental gradients (e.g., latitudes or depths), within and across taxa.

To gain deeper insights into the genetic basis of elevated thermal tolerance, highly resistant species or populations from extreme habitats should be included and compared to conspecifics from less particular locations in close proximity. For example, the exceptionally heat tolerant species *Pararotalia calcariformata* (Schmidt

et al. 2016b), which even continues to calcify at temperatures as high as 40°C (Titelboim et al. 2017), or populations from thermally variable and extreme habitats such as tidal rock pools (Weinmann and Langer 2017) or shallow-water hydrothermal vents (Engel et al. 2015) could represent valuable test groups. Further symbiosis-related characteristics that should be considered in experimental studies are the ability of diatoms and other endosymbiotic algae to alter their pigment compositions, as well as the ability of the host to shuffle between symbionts (i.e., the shift to different preferential symbiotic partners). Moreover, such studies should include the influence of several synchronously and also successively acting multiple stressors.

To continue elucidating the underlying cellular mechanisms of adaptive responses, reciprocal transplant experiments should be conducted that expose LBF originating from highly fluctuating environments into more stable neighboring habitats and the other way around (Barshis et al. 2010; Mayfield et al. 2012; Ziegler et al. 2017). While this approach is relatively straightforward with corals, monitoring and re-collecting the same LBF specimens over extended time periods is a major obstacle, but can potentially be achieved by using flow-through containers or mesh bags (Engel et al. 2015). Alternatively, LBF can be brought to experimental facilities that realistically mimic the differing environmental conditions. To gain maximum insights into holobiont characteristics contributing to resilience, different host and symbiont lineages need to be included, possibly encompassing variations in genotypes that are adapted to different thermal ranges (Barshis et al. 2010; Ladner et al. 2012). Besides employing traditional methods to estimate symbiont and host, as well as holobiont performance, omics approaches such as proteomics offer a variety of additional benefits. These will give meaningful insights into the molecular responses that are fundamental for differences found in adaptive capacities, as illustrated by mostly transcriptomic studies on corals (Mayfield et al. 2012, 2013, 2016a; Palumbi et al. 2014; Bay and Palumbi 2017; Ruiz-Jones and Palumbi 2017). Additionally, this could lead to the establishment of cellular biomarkers and contribute to predicting the persistence of LBF under future climate change.

6.6 Further food for thought on experimental designs

Recovery is an important component of resilience (Baker et al. 2008; Thomas and Palumbi 2017) and should therefore included into study designs. Future experiments should not be ending with the termination during or directly after stress exposure. In stead, retaining subsets of specimens could be used to monitor physiological parameters and cellular mechanisms of recovery (Thomas and Palumbi 2017), as well as hypothetical re-admission and shuffling of symbionts.

To include possible trade-offs of enhanced stress resistance, the timing and reproductive cycles of the experimental populations need to be taken into account

during project planning. Although reproduction during experiments can denote a challenge for data evaluation of parent physiology, impacts on fecundity and reproductive success are important factors than can have severe consequences on the LBF community level and hence represent meaningful proxies of holobiont response. Along these lines, multigenerational experiments of clonal cultures of relatively fast reproducing taxa such as diatoms and foraminifera comprise the potential to detect genetic adaptation in practicable timescales. These valuable opportunities should be explored in forthcoming studies.

Environmental stress and the resulting effects on LBF calcification and growth may not always be detectable by reductions in surface area, but can also be expressed as malformations (Fig. 6-2) or reductions in test density (Toler and Hallock 1998; Prazeres and Pandolfi 2016). To include such impacts into monitoring and experiments, shell density of the entire organism can be scanned and modeled using computer tomography (Fig. 6-3a). Alternatively, growth of added chambers can be precisely measured on thin-sections of stained specimens. By adding the fluorescent probe calcein to the culture (Erez et al. 2010), e.g., at the beginning of an experiment, which is then incorporated into the newly produced layers of calcite (Fig. 6-2b), one can determine the number, size and thickness of new chambers.

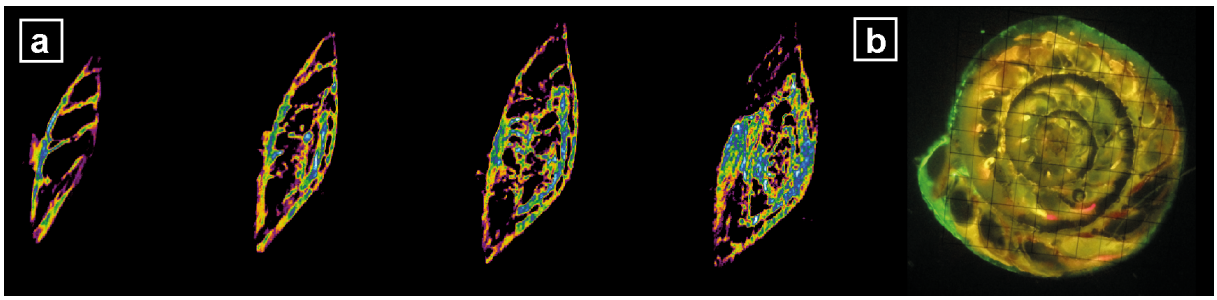

Figure 6-3. Vertical and horizontal sections through Amphistegina*: a) slices of a computer tomography reconstruction of* A. gibbosa *(credit: T. Junginger). Colors indicate density of the calcite test from purple (low) to blue (high); b) thin-section of* A. lobifera *recently stained with calcein. Bright green areas on the outer chambers specify freshly produced calcite.*

Lastly, another factor that may influence the resilience of photosymbiotic reef organisms is the assemblage of associated microorgansims. The coral microbiome can react quickly to thermal stress and, as this response differs between corals of distinctive heat susceptibility, this might play an important role for heat tolerance (Ziegler et al. 2017). Also in other coral reef calcifiers, including LBF, the microbial community is sensitive to higher temperatures and shifts in response to environmental stressors (Webster et al. 2016). *A. lobifera* populations collected from different habitats across the Great Barrier Reef form specific associations with prokaryotic microbes, depending on sampling site (Prazeres et al. 2017a). As these populations were formerly shown to differ in physiological responses to environmental stressors (Prazeres et al. 2016b, 2017b), not only variations in their

eukaryotic endosymbionts (i.e., microalgae), but also their bacterial associates may be involved in different adaptive capacities (Prazeres et al. 2017a). Consequently, to understand the potential role of symbiotic interactions in the adaptive responses of organisms, we should not only aim at elucidating interactions between hosts and their known photosymbiotic partners (if given), but widen our perspectives by incorporating the variety of other potentially critical interactions with prokaryotic and non-photosynthesizing eukaryotic partners.

An increasing understanding of the evolutionary forces that shape symbiotic associations and their resilience mechanisms can help identify key taxa and potential refugia, develop high-resolution biomarkers, and contribute to efficient conservation management to mitigate detrimental effects of environmental change.

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