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Dormancy in the *Amphistegina gibbosa* Holobiont: Ecological and Evolutionary Implications for the Foraminifera

Benjamin J. Ross
University of South Florida, ben.ross8@gmail.com

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by

Benjamin J. Ross

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Major Professor: Pamela Hallock Muller, Ph.D.
James Garey, Ph.D.
Kendra Daly, Ph.D.
Mya Breitbard, Ph.D.
Susan Richardson, Ph.D.

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DEDICATION

This dissertation is dedicated to my dad, the Doctor I always wanted to be, and to my mom, who helped me to get there.

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ABSTRACT

Dormancy, a state of severely decreased or suspended metabolism, is a widespread survival strategy in nature. In the Foraminifera, one of the most studied groups of marine organisms, its presence had been suggested by circumstantial evidence, but rarely studied directly until recently. Despite the lack of research, stressor-induced dormancy can significantly alter the way in which foraminiferal ecology is understood, especially in marginal environments. In this dissertation, I reviewed the evidence for dormancy in the foraminiferal literature, concluding that evidence for dormancy is widespread across the Phylum. I then explored the role of dormancy in the survival of the diatom-bearing foraminifer *Amphistegina gibbosa* d'Orbigny when exposed to toxic chemicals, and when kept in dark conditions for extended periods of time. I developed methods for utilizing CellTracker Green™, a fluorescent probe, to explore metabolic activity in symbiont-bearing foraminifers, finding that it can be used in some situations, such as bioassay experiments or other cases of toxic chemical exposure, to distinguish dead from dormant individuals. The results of the associated experiments demonstrated that reduced metabolism occurred in individuals that survived toxic chemical exposure for over two months in darkness, as well as indicating that metabolic recovery can begin to occur within 30 minutes of removal from darkness. Fluorescence microscopy of symbiont autofluorescence also demonstrated that the diatom symbionts are also capable of surviving aphotic conditions, recovering when returned to lighted conditions.

Further experiments showed that *A. gibbosa* and its associated symbionts are capable of surviving up to 20 months in darkness. Although survival decreased as the length of time in darkness increased, 80% of the specimens survived a 20-month treatment. In addition, all treatment lengths showed color recovery, indicating survival of the diatom symbionts, which give *A. gibbosa* its characteristic golden-brown color. However, patterns of color recovery indicated that extended periods in darkness increased the photosensitivity of the *A. gibbosa* holobiont, despite entering dormancy.

CHAPTER ONE

INTRODUCTION

Rationale

Foraminifera are one of the most studied groups of marine organisms. They are utilized in a variety of applications, including in paleoceanographic and paleoenvironmental reconstructions, economic geology such as oil exploration, and as bioindicators, especially in coastal zones. Many of these applications rely on an understanding of foraminiferal biology and ecology to interpret the patterns and geochemical signatures that can be read from individuals and assemblages.

Ross & Hallock (2014) developed methods to use the Caribbean reef-dwelling, symbiont-bearing larger benthic foraminifer *A. gibbosa* as a bioassay organism for studying the effects of chemicals on reef-dwelling benthos, including corals. In the process of developing these methods, we discovered that *A. gibbosa* was able to survive exposure to propylene glycol and 2-butoxyethanol by going dormant. They would cease all activity and, without allowing recovery, appeared functionally dead. Following a recovery period [72 hours in Ross & Hallock (2014)], however, many of these inactive individuals would resume normal activity.

Many of the applications for which foraminifers are employed rely on at least an implicit understanding of how foraminifers react to environmental perturbations and stressors. Paleoceanographic applications, for example, interpret fossil foraminiferal populations through the lens of observable modern foraminiferal behavior and ecology to draw conclusions about

past environmental conditions. Economic geology, such as oil exploration, correlates fossil foraminifers to biostratigraphic or paleoenvironmental conditions favorable for oil production. The use of foraminifers as bioindicator species requires an understanding of how they react to stressors to understand the environmental conditions that they reflect. The ability to go dormant in reaction to stressors could alter interpretations of foraminiferal assemblage patterns, and have wide ranging implications across all of these study areas. To explore this adaptation, this dissertation reviews evidence for dormancy in the literature and presents a number of experiments exploring dormancy in *A. gibbosa* specifically.

Organisms of Study

Amphistegina gibbosa d'Orbigny is a larger benthic foraminifer of the Order Rotalida, Family Amphisteginidae. The genus *Amphistegina* can be found nearly circumtropically at depths down to 120 meters (Hallock, 1999; Langer & Hottinger, 2000). *Amphistegina gibbosa* is the primary species of the genus present in the Caribbean and western Atlantic (Hallock, 1988a,b; Williams, 2002). *Amphistegina* spp. are known to host diatom endosymbionts (Lee et al., 1995; Barnes, 2016) which, when the foraminifer is healthy, occur in pore cups along the periphery of the shell chambers within the cellular endoplasm (Talge & Hallock 1995, 2003).

When exposed to photooxidative stress, the host foraminifer will digest the endosymbionts and surrounding cytoplasm, leading to foraminiferal "bleaching" as color from the diatom symbionts is lost (e.g., Hallock et al., 1992; Talge & Hallock, 2003). This color loss is closely related to irradiance in the field (Williams et al., 1997). In the laboratory, signs of photic stress have been observed at relatively low irradiances (Hallock et al., 1986; Williams & Hallock, 2004). This has made *A. gibbosa* useful bioindicators for threats to coral reefs, due to

the similarity to the bleaching response in corals (e.g., Hallock et al., 2006). This prompted the development of methods to utilize *A. gibbosa* as a bioassay organism relevant to understanding the effects of chemical exposure on the coral and associated benthos on coral reefs (Ross and Hallock, 2014). During this methodological development, I discovered that *A. gibbosa* were capable of going dormant to survive chemical exposure.

The *Amphistegina*-symbiont holobiont is obligately photosynthetic (Hallock, 1999, and references therein). This complicates the interaction between host and symbiont in terms of stress responses, as high irradiance can cause photic stress and damage, while lack of light inhibits growth of the host. In the field, *A. gibbosa*'s phototaxic capabilities help modulate light exposure (e.g., Zmiri et al., 1974; Sinutok et al., 2013), but complete lack of light precludes photosynthesis. Despite this, *A. gibbosa* were previously observed to survive up to 12 months in aphotic conditions (Smith & Hallock, 1992), interpreted to be the result of a reduced-metabolism, dormant state.

Toxic-chemical exposure and darkness are known initiators of dormancy in *A. gibbosa*. Moreover, this species has documented utility as a bioindicator and bioassay organism, which requires an understanding of stress responses. The species is also amenable to culture environments. Thus, *A. gibbosa* is an ideal candidate for exploration of dormancy in the Foraminifera in general, and in symbiont-bearing, larger benthic foraminifers in particular.

Major Questions

a. How widespread is evidence for dormancy across the Foraminifera?

b. Can methods such as the use of CellTracker™ Green (CTG) provide alternative indicators of activity of the foraminifers to effectively differentiate between dead and dormant individuals?
c. Can CTG be used to recognize differences in metabolism between dormant and non-dormant foraminifers?
d. How long can *Amphistegina gibbosa* and symbionts survive darkness?
e. Is there a difference in the survival potential of host and symbiont during dormancy (e.g., during darkness)?

Overview of Dissertation

In addition to this Introduction, this dissertation is composed of four chapters with a central theme of dormancy within the Foraminifera, with a particular focus on *Amphistegina gibbosa*. The final chapter presents overall conclusions and recommends future directions for research into the physiology of dormancy.

- Chapter 2 is a comprehensive literature review summarizing evidence for dormancy in the Foraminifera drawn from decades of literature, identifying its presence across taxa and drawing conclusions concerning its ubiquity across the phylum. This paper has been published in the *Journal of Foraminiferal Research* (Ross & Hallock, 2016).
- Chapter 3 describes the use of the fluorescent probe CellTracker ™ Green CMFDA to identify changes in metabolic activity related to dormancy in *Amphistegina gibbosa*. This paper has been published in the open-access journal *PeerJ* (Ross & Hallock, 2018).

- Chapter 4 describes the survival ability of the *Amphistegina gibbosa* holobiont (the combination of foraminiferal host and diatom endosymbionts) in extended conditions of total darkness.
- Chapter 5 uses statistical methods to further assess patterns of survival, and uses conclusions from Chapters 2, 3 and 4 to provide a clearer picture of survival under aphotic conditions, with a focus on the evidence for gradients of survival.
- Chapter 6 provides a summary of the results and a synthesis of the data from the previous chapters, while highlighting research involving dormancy in the Foraminifera published after the publication of Chapter 2, and suggesting further directions for the study of dormancy in the Foraminifera.

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CHAPTER TWO

DORMANCY IN THE FORAMINIFERA: A REVIEW

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CHAPTER THREE

CHALLENGES IN USING CELLTRACKER GREEN ON FORAMINIFERS THAT HOST ALGAL ENDOSYMBIONTS

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CHAPTER FOUR

SURVIVAL AND RECOVERY OF THE FORAMINIFER *AMPHISTEGINA GIBBOSA* AND ASSOCIATED DIATOM ENDOSYMBIONTS FOLLOWING UP TO 20 MONTHS IN APHOTIC CONDITIONS

Abstract

Dormancy in the Foraminifera has been observed widely across the phylum in reaction to a variety of triggers including, in the diatom symbiont-bearing foraminifer *Amphistegina gibbosa*, extended periods of darkness. Previous research observed recovery of activity in the host-symbiont holobiont following up to 12 months in darkness. Here, holobiont recovery of 100% of the sample population following up to 12 months in darkness, and over 80% of the sample population following up to 20 months in darkness, was documented. Image analysis using the percent of the foraminiferal surface area showing color as a proxy for symbiont recovery showed continued recovery over time for shorter treatments (7 and 12 months in darkness), but less recovery, and possibly loss of color over time in longer treatments (15 and 20 months), which may indicate increased susceptibility to photic damage of symbionts as the length of dormancy increases.

Introduction

Dormancy describes a life-history stage with a wide variety of initiating triggers and modes of expression. Dormancy commonly involves the suspension of active life, arrested development, reduced or suspended metabolic activity, and the ability to recover from these conditions (i.e., Càceres, 1997; Guidetti et al., 2011; Lennon & Jones, 2011). Some manifestation of dormancy is found across a wide variety of taxa, including plants, mammals, fish, and reptiles (see Ross & Hallock, 2016, and references therein) and especially among protistan groups, including marine protists such as dinoflagellates (e.g., Binder & Anderson, 1990; Figueroa et al., 2007; Smayda & Trainer, 2010; Lundholm et al., 2011; Bravo & Figueroa, 2014) and diatoms (e.g., Smetacek, 1985; McQuoid & Hobson, 1996; Lewis et al., 1999; von Dassow & Montresor, 2010). O'Farrell (2011) considers cellular quiescence to be a fundamental, primitive adaptation to survive resource limitations inherent to rapid generation times; its presence in "primitive" single-celled organisms is nearly ubiquitous.

Members of the protistan Phylum Foraminifera (d'Orbigny, 1826) (phylum status as proposed by Mikhalevich, 2004 and Pawlowski et al., 2013) are a ubiquitous group in marine environments and even some terrestrial ones. Historically research has focused on the shells of dead foraminifers due to the preservation potential of their organic or agglutinated tests or calcareous shells in Phanerozoic sediments and sedimentary rocks (e.g., Sen Gupta, 1999). Because of this preservation potential, as well as their abundance (second only to coccolithiphores as a component of calcareous sediment) (Kennett, 1982), foraminiferal shells are an integral component to the study of the geologic past and paleoceanographic conditions, as well as having many economic applications. Because of the utility of preserved shells, research

has historically been focused on dead foraminifers (e.g., Sen Gupta, 1999). Goldstein (1999) estimated that, of >10,000 extant species, relatively complete life cycles are known for <30, despite the common use of modern analogue species to interpret the paleoenvironments of fossil assemblages.

Thus, few studies have directly addressed dormancy in the Foraminifera. Nevertheless, a review of foraminiferal research concluded that dormancy appears to be a common adaptation across this phylum as well (Ross & Hallock, 2016). Dormancy has been suggested as a survival response to a number of environmental triggers in the Foraminifera, including temperature (Bradshaw, 1957), anoxia (Bernhard, 1993; Bernhard & Alve,1996; Moodley et al.,1997), anoxia with accompanying reducing conditions (Bernhard, 1993; Langlet et al., 2013, 2014), toxic chemical exposure (McCloskey, 2009; Ross & Hallock, 2014, 2018), and extended darkness in photosymbiotic species (Smith & Hallock, 1992). Darkness as a trigger for dormancy is interesting because one of the species studied, *Amphistegina gibbosa* d'Orbigny, is a common indicator species on Caribbean coral reefs. Their shells are a component of the FoRAM (Foraminifera in Reef Assessment and Monitoring) Index, a single-metric index of water quality developed for use in the western Atlantic and Caribbean, and live populations have been used as a relatively quick, low-cost method to assess environmental conditions on a reef to determine if stressors are emerging (Hallock et al., 2003; Spezzaferri et al., 2018).

Amphistegina spp. are relatively large, shelled foraminifers, abundant in warm seas nearly circumtropically, living primarily on phytal and hard substrata in coral-reef and open-shelf environments. *Amphistegina* host diatom endosymbionts in an obligate relationship similar to that of zooxanthellate corals (e.g., Lee, 2006 and references therein), including bleaching (loss of color as a result of loss of algal symbionts, e.g., Hallock et al., 1995; Williams et al., 1997,

Williams & Hallock, 2004), which is in part why they are such useful indicators of conditions on reefs (e.g., Spezzaferri et al., 2018).

Much of this utility, however, depends on an understanding of the host-symbiont relationship and its light requirements. Lee et al. (1991) found that *Amphisorus hemprichii* Ehrenberg (a dinoflagellate- bearing miliolid) and *Amphistegina lobifera* Larsen (a diatom-bearing rotaliid) survived for 8 and 13 weeks in total darkness, respectively. Smith & Hallock (1992) reported on the survival of *Archaias angulatus* (Fichtel & Moll) (a chlorophyte-bearing miliolid) and *Amphistegina gibbosa* held in total darkness, observing that some individuals survived up to 3 and 12 months in darkness, respectively. The surviving *Am.gibbosa* subsequently regained normal symbiont color and behavior after being returned to a normal day/night light cycle. The ability to become dormant under prolonged darkness has wide-ranging implications for interpretations of foraminiferal assemblage data. For example, individual specimens of taxa that live epifaunally can be buried by hydrodynamic disturbance or bioturbation of sediments. If such individuals can survive accidental burial by entering a dormant state, recognizing this possibility has many implications for both modern and paleoenvironmental studies.

Recovery of both host activity and symbiont color implies co-dormancy between the host and algal symbionts. Understanding the coordination of this relationship could have widespread implications, including in medicine. For instance, some of the most damaging human illnesses are caused by parasites (e.g.,, *Plasmodium* spp., which cause malaria), which often have complex life histories that include dormancy. Understanding the drivers of co-dormancy could help understand how these parasites can persist in human hosts (i.e. dormancy in quiescent cells).

Unfortunately, the initial work by Smith & Hallock (1992) was only reported in an abstract, leaving a literature gap concerning this life-history strategy in foraminifers that host algal symbionts. The goals of my study were to follow up on this earlier research, focusing on *Amphistegina gibbosa*. I hypothesized that some recovery would occur after at least 12 months in darkness as reported by Smith & Hallock (1992). I also hypothesized that recovery would significantly decrease thereafter.

Materials and Methods

Individual *Amphistegina gibbosa* were picked under a stereomicroscope from reef rubble collected from 18 m water depth at Tennessee Reef in the Florida Keys (24.7523°, –80.7549°). Groups of five individuals were placed in microcentrifuge tubes (1.5 ml) with pierced holes at the top and bottom to allow for water and gas exchange. These tubes were then placed in sealed, semi-opaque Nalgene® containers. The containers were filled to the top with seawater collected at Tennessee Reef, then wrapped in two layers of aluminum foil and placed in an incubator at a constant temperature of 25°C. Individuals were held in complete darkness and sampled at 7, 12, 15, and 20 months. Two sets of experiments were established five months apart, allowing 7 and 12 month treatments to be sampled on the same day, as were the 15 and 20 month treatments.

Based on substantial experience with laboratory experiments and field studies over the past >30 years, *A. gibbosa* is well known to be sensitive to photo-oxidative stress (e.g., Hallock et al., 1986; Williams & Hallock, 2004), especially during photographic documentation (Ross & Hallock, 2018). To focus on recovery potential while minimizing photic stress, individuals were introduced to a full night/day light cycle gradually. In the earlier experiment (Smith & Hallock,

1992), specimens were moved directly from darkness into normal culture conditions of ~10-15 µmol photons m^{-2} s^{-2}.

For this experiment, extreme care was taken to limit photic stress during the first few days of reintroduction to light. When removing replicate tubes for observation, the holding containers were opened in a darkened room with minimal light (< 1 µmol photons m^{-2} s^{-2}).When not actively removing tubes, the containers were kept under a box wrapped inside and out with opaque black plastic; no measureable presence of light could be detected within the box, even in full ambient laboratory light (~6 µmol photons m^{-2} s^{-2}). Closed tubes were rapidly removed from containers and temporarily placed in 5 ml well plates covered in a double layer of mesh. The holding containers were closed, re-wrapped in aluminum foil and returned to the incubator, again to minimize light exposure.

Tubes and well plates were moved to the main laboratory space, where only ambient light from the windows entered the room (~2 µmol photons m^{-2} s^{-2} maximum). The seawater from each tube was poured into a labeled well plate, then the foraminifers were removed from the tube and placed in that well plate using a soft brush. The original seawater was removed via pipette and replaced with new seawater collected from the same location. When not being directly manipulated, well plates and tubes were kept under mesh to minimize light exposure.

After being removed from the tubes, all individuals were examined for evidence of vital activity under a dissecting stereoscope; if additional light was necessary to observe the specimens, the lowest intensity was used (~12–30 µmol photons m^{-2} s^{-2} maximum). Individuals were considered “active” if they exhibited visible waste material production, visible extrusion of granuloreticulopodia, attachment to the sides or bottom of the well, or were found floating along the surface of the water after climbing the sides of the well. Such activity was considered

indication that an individual was alive and not dormant. When vital activity was not immediately discernible, individuals were lightly manipulated with a soft brush to determine if attached. Care was taken to not detach individuals, to minimize damage to extruded granuloreticulopodia, which might have affected recovery.

A separate set of replicates at each time interval was set aside for photographic analysis of color. In the 7- and 12-month observations, three sets of five individuals each were photographed. For the 15- and 20-month samples, only one set of five individuals was available (following removal of sets for use in other observations). These specimens were photographed daily for the first 5 days, then photographed less frequently up to 16 days post-removal, then again at 34 days post removal for the 7 and 12 month treatments, and 18 and 28 days post removal for the 15- and 20-month treatments. Photography exposed the foraminifers to light levels ~20 µM photons m^{-2} s^{-2}.

These photographs were assessed for evidence of color recovery over time by analyzing the percent of the visible surface area of each individual foraminifer that is colored, indicating the presence of symbiont-bearing endoplasm. Image analysis was performed using ImageJ. Due to the presence of white chamber walls visible through the outer wall of the shell, automated threshold-based binarization transformations did not reliably reveal differences in color across the surface area of the shell. As a result, brightness and contrast were adjusted for each individual foraminifer to emphasize the colored regions, and then a Phansalkar transformation was applied to binarize the colors into black and white, and the percentage indicating presence of color measured using ImageJ tools.

For the 7- and 12-month treatments, samples were left to recover on a lab bench beneath a window, so as to receive naturally variable light. Laboratory temperature was 23–25°C, and

light levels varied from < 1 µmol photons m^{-2} s^{-2} at night with the laboratory lights off, to ~7 µmol photons m^{-2} s^{-2} during the afternoon when light directly entered the windows, and the overhead lights were on. For the 15- and 20-month treatments, technical issues resulted in laboratory temperatures of ~17°C, so samples were allowed to recover in an incubator with ambient light levels (depending on positioning, which was rotated after daily sampling) of ~3–7 µmol photons m^{-2} s^{-2} on a 12-hour light/dark cycle at 25°C. To minimize photic stress after being held in the dark for such long periods, the samples were initially covered in a double layer of light-attenuating mesh for two days, which decreased light levels by 80%; a single layer of mesh for another two days, which decreased light levels by 40%; and then a return to full ambient light levels, either on the lab bench or within the incubator. Observations were made every day as close to 24 hours following the previous observation as possible. Water in all well plates was changed every 48 hours.

All statistics were calculated using either MATLAB with the Fathom toolbox (Jones, 2015) or Microsoft Excel with the Real Statistics Resource Pack software (Release 5.4, Copyright, 2013–2018, Charles Zaiontz. www.real-statistics.com). For activity recovery, data for each day of observation were compared using a non-parametric, dissimilarity-based repeated-measure MANOVA [f_NPManova (Jones, 2015)] in MATLAB. Because the test requires a balanced design, and because only nine replicates were assessed for the 7- and 15-month treatments, while 10 replicates were assessed for the 12- and 20-month treatments, a tenth data point was added by calculating the mean of all other replicates. Similarly, because sampling could not be performed on the same schedule between the two shorter treatments and the two longer treatments, days post-removal on which all four treatments were not sampled were

removed from this initial comparison, with the exception of 28 and 33 days, which were grouped together to represent longer term recovery.

Comparisons of the rates of recovery were performed by first isolating the days on which linear recovery occurred (prior to the asymptotic portion of the curve, see Fig. 4.1). This was done via pairwise t-tests assuming unequal variance and identifying the first day that did not significantly differ from the next; the earlier day was considered to be the end of the linear recovery curve. Figure 4.1 visualizes this difference between a period of rapid recovery, followed by a consistent plateau. Comparisons of the slopes were performed using a nonparametric Analysis of Covariance test [f_Ancova (Jones, 2015)] in MATLAB.

For the color recovery experiment, imbalances in time of sampling and sizes of samples necessitated the use of a non-parametric, dissimilarity-based repeated-measures MANOVA [f_NPManova (Jones, 2015)] in MATLAB, which allows for the use of imbalanced designs; averages of daily averages per replicate were used for this analysis.

Results

Nearly all experimental specimens showed resumption of activity in the 7- and 12-month treatments, and ~80% of specimens from the 15- and 20-month treatments survived and exhibited recovery when gradually reintroduced to pre-treatment light conditions (Figs. 4.1, 4. 2). A scatter plot of daily percent recovery (Fig. 4.1) revealed similar distributions between 7- and 12-month treatments, and similarity between 15- and 20-month treatments, with notable differences in both slope and asymptote between the groups. The asymptotes were reached by day 3 for the 7- and 12-month treatments, and by day 5 or 6 for the 15- and 20-month treatments. The 15- and 20-month treatments also exhibited higher variability.

Comparisons of resumption of activity between the results of the different treatments using a 2-way Repeated Measures ANOVA with no replication revealed significant differences among the treatments (factor 1 and 2, $p = 1x10^{-3}$). Pairwise testing (Table 4.1) revealed significant differences only between the 7- and 20-month treatments ($p = 0.02$). Comparisons of the slopes generated from activity observations (Table 4.1) revealed significant differences between results for 7- and 15-month treatments ($p = 0.02$), for 7 and 20 months ($p = 0.001$), 12 and 15 months ($p = .001$), and 15 and 20 months ($p = 0.002$).

Comparisons between recovery of color based upon percent surface area of the foraminifers over time (Fig. 4.3, Table 4.1) at a p-value of 0.05 did not show a significant difference between 7 and 12 months ($p = 0.6$), but did show differences between all other treatment pairs: 7 and 15 months ($p = 0.003$), 7 and 20 months ($p = 0.001$), 12 and 15 months ($p = 0.004$), 12 and 20 months ($p = 0.002$), and 15 and 20 months ($p = 0.02$).

Figure 4.4 shows a comparison of this color recovery over time between treatments in representative small, medium, and large individuals from each treatment. This comparison shows that, even in the healthiest looking individuals at 30 days in the 15- and 20-month treatments (which had not been photographed daily), white spots ("mottling") can be seen. In the 15-day treatments, which had been photographed daily for the first 8 days, and then every other day, the white spots devoid of surface color were even more pronounced, especially in comparison to the individuals in the 7- and 12-month treatments.

Discussion

This study demonstrated that *Amphistegina gibbosa* holobionts, specifically the foraminiferal host and its diatom endosymbionts, are capable of surviving and recovering from

periods of total darkness for up to 20 months, with 100% recovery observed in treatments kept in darkness for 12 months. These findings differ from those of Lee et al. (1991), who observed drastically shorter lengths of survival of both *Amphisorus hemprichii* and *Amphistegina lobifera*, calling into question the ubiquity of this survival mechanism, even within the same genus. However, Lee et al. (1991) removed and observed their specimens weekly; it is possible that being regularly returned to light may have decreased the long term efficacy of darkness-induced dormancy. These results also differ from those of Smith & Hallock (1992), who reported "some" recovery after 12 months, and no recovery of foraminiferal activity after 18 months in total darkness. Unfortunately, the original data from this previous study has been lost, thereby limiting comparisons. I observed that the plateau in recovery may take up to 5 days to reach, and no mention is made in the published abstract as to how long specimens were observed post-removal.

A possible explanation for the unexpectedly high percentages of recovery observed in my experiments was the light regime used following removal from darkness. Recognition of the sensitivity of individual *Am. gibbosa* to photic stress has greatly increased since the Smith & Hallock (1992) study (e.g., Hallock et al., 1995; Talge & Hallock, 2003; Williams & Hallock, 2004). In particular, recent fluorescence experiments and photographic documentation thereof (Ross & Hallock, 2018) demonstrated that even brief exposure to high light during photographic documentation can compromise survival of experimental specimens. Thus, extreme care was taken to limit light exposure during handling and examination, and to only gradually reintroduce the experimental specimens to even the very low light intensities routinely used in laboratory experiments, to prevent partial bleaching (e.g., Hallock et al., 1986). Return to full light immediately could be stressful for dark-adapted individuals. This was intended to mimic the phototaxic capabilities of *Am. gibbosa* (described in *Amphistegina* spp. in Zmiri et al., 1974),

which is limited in well plates and petri dishes devoid of hard, opaque cover such as coral rubble, but allows the foraminifers to control their level of light exposure in the field (see also Sinutok et al. 2013). As a result, limitation of photic stress during reintroduction to light apparently allowed the sensitive foraminifer-symbiont holobiont to recover from long-term darkness, and may have enabled the survival of the foraminifers much longer than that observed by Smith & Hallock (1992).

The ability of *Am. gibbosa* to adapt to light regimes at a variety of depths (Hallock, 1999) may also play a role. Smith and Hallock (1992) provide no indication of the depth at which specimens were collected. The specimens used in this experiment were collected at 18 m, although they had been acclimated to laboratory conditions before the start of their dark incubation.

Although such gradual reintroduction to light was intended to allow for maximum observable recovery, recovery under very low light is quite possible in the field, especially at depths >20m, where light intensities are much lower than at shallower depths (i.e., Williams et al., 1997; Mendez-Ferrer et al., 2018). For example, *A. gibbosa* are known to occur on the Flower Garden Banks at >29 N latitude and depths in excess of 50m (e.g., Poag, 2015, and references therein). Combined with phototaxic behavior (Zmiri et al., 1974; Sinutok et al., 2013) that allows individuals to become more cryptic in higher light conditions, light exposure in natural habitats may not differ as much as could be expected considering the depth ranges over which *A. gibbosa* have been found living (e.g., Barnes, 2016). Dormancy in darkness could facilitate survival when buried for months to a few years, and would allow populations of foraminifers that host algal endosymbionts to survive extended periods of low light availability. Examples might include mid- and outer-shelf depths where monsoon conditions result in

seasonal increases in water turbidity and therefore limit depth of light penetration. Similarly, species with algal symbionts might survive seasonal changes in light penetration by becoming dormant. *Amphistegina* and other species of larger benthic foraminifers occurred at latitudes as high as 50° in warmer ocean waters during the Paleogene (e.g., Todd, 1976).

Sensitivity to exposure to higher light regimes was further demonstrated by the color recovery data (Fig. 4.3). The 7- and 12-month treatments showed a gradual increase in colored surface area over 11 days of repeated photography. The 15- and 20-month treatments, on the other hand, show a gradual reduction in colored surface area following 12 days of repeated photography. The final photographs of both treatments (Fig. 4.3) were taken of the previously unphotographed replicates used for activity recovery observations; colored surface area in these specimens was visibly higher than in specimens repeatedly photographed. This suggests that the decrease in area of color was the effect of damaging light exposure during photography, as well as suggesting that longer periods in darkness may make individuals more sensitive to light than shorter periods.

In addition, as shown in Figure 4.4A–B, smaller individuals (< 0.5 mm) seem to recover more quickly and more fully. This may be because, upon removal, any color present tends to be concentrated in the center of the shells (Fig. 4.4A). Whether this indicates concentration of all cytoplasm and organelles in the interior chambers, or just a movement of the diatom symbionts to the center, color recovery starts deep within the shell. In smaller individuals, refilling the outer chamber apparently can happen more quickly.

The colorless spots observed in some specimens post-recovery could be related to localized damage associated with the stress of the aphotic conditions. Talge & Hallock (1995) observed that *Am. gibbosa* may be able to partition and isolate damaged chambers. If part of the

dormancy survival mechanism involves isolating damaged chambers, for instance, chambers in which cytoplasm is digested for survival, then these chambers may remain isolated post-recovery as indicated by the colorless portions of the shells (e.g., Fig. 4.4C).

Another pattern seen in the activity recovery (Figs. 4.1, 4.2) was that the timing and extent of recovery differed significantly between the 7- and 20-month treatments, but not between other pairs; this suggests a gradual change in survival as treatment length increases. This result likely reflects the extreme care taken in this study to minimize photic shock.

However, the rate of recovery decreased between 12 and 15 months. In the 7 and 12 month treatments, recovery plateaued after 3 days, which was at the end of the first day in which the specimens were covered by a single layer of light-attenuating mesh. In the 15- and 20-month treatments, the plateau in recovery required 5 days, which was at the end of the first day of full ambient light.

These differences between the 7- and 12-month treatments compared to the 15- and 20-month treatments were complicated by the necessary change in recovery location. However, the samples kept in the incubator were exposed to more consistent temperatures (25°C in the incubator compared to 23–25°C in the laboratory) and light conditions, and longer periods of light per day. In addition, maximum light levels are approximately the same as on the lab bench (~7 µmol photons m^{-2} s^{-2}). Thus, the 15- and 20-month treatments likely were not at a disadvantage in recovery conditions in general. However, removal from the incubators each day to observe the specimens under the microscope did submit the 15- and 20-month treatment specimens to cooler air temperatures for approximately 30-45 min per day during the initial days of observations. However, the greater variability seen in the 15- and 20-month treatments (see

error bars in Figure 1) supports the conclusion that recovery was not as consistent in the longer treatments.

Regardless of the timing of recovery, the discovery that recovery is possible after almost two years in darkness has important implications for the ecology of these foraminifers and for paleoecological interpretations. This observation indicates that a form of co-dormancy could play an even more significant role in the survival of *Am. gibbosa* in aphotic conditions than suggested by previous research. If foraminifers that have become buried (i.e., due to storm conditions or bioturbation) are capable of recovering if returned to photic conditions up to at least 20 months post-burial, this survival ability may contribute to the relatively rapid reestablishment of populations following large disturbances, in much the same way that the cryptic propagule "seed bank" (i.e., Alve & Goldstein, 2002, 2003, 2010; Goldstein & Alve, 2011)] is thought to do. It could also explain the presence of live-staining foraminifers found infaunally during sampling, which might otherwise be dismissed as post-mortem staining (i.e., with rose bengal). Finally, these observations could have implications concerning the range expansion of *Am. gibbosa,* and potentially other symbiont bearing taxa, as global temperatures continue to rise. Weinmann et al. (2013) noted that symbiont-bearing foraminifers are primarily temperature limited and that populations show poleward expansion in times of warmer seas. Using Species Distribution Models, Weinmann et al. suggested significant poleward expansion will occur under predicted climate warming trends. Similarly, Langer et al. (2013) used these techniques to predict expansion of *Amphistegina* spp. specifically, predicting a 264 km southward expansion along the African coast by 2100. In warmer climates in the geologic record, Todd (1976) reported *Amphistegina* species in the Miocene at latitudes as high as 50° N in the Vienna Basin and Poland, and to 37° S in Australia. In the Eocene, *Amphistegina* occurred as far north as 48° N on

the Olympic Peninsula in Washington, USA, and as far south as 36° S in New Zealand (Todd, 1976).

Light is a necessary component of the trophic strategy of photosymbiont-bearing foraminifers, and light is more seasonally variable at higher latitudes. Ross & Hallock (2018) used CellTracker Green incubations to observe recovery of *Am. gibbosa* after a shorter 6-week darkness period. Results indicated that recovery may begin within 30 minutes of being returned to lighted conditions, thus photoperiodic dormancy could act on short enough time scales to account for seasonal variations in day length. This could be an important factor in understanding range expansion both in the geologic record and in the near future. *Amphistegina lessonii* and *Am. lobifera*, for instance, are highly successful Lessepsian invasives in the Mediterranean Sea, where they have both disrupted native foraminiferal biota (e.g., Langer et al., 2012) and altered coastal sedimentation (Triantaphyllou et al., 2009, 2012). Global range expansion could have similar effects in other areas, and understanding all of the factors that allow for expansion will help to predict the effects of this expansion as the oceans warm.

Symbiosis is a key adaptation in larger benthic foraminifers (i.e., Hallock, 1999, and references therein). *Amphistegina* spp. host diatom symbionts, and diatoms are well known to be able to survive in a dormant state for prolonged periods of time (i.e., Jewson et al., 1981; Sicko-Goad et al., 1989; Itakura et al., 1997; Ribeiro et al., 2011). My observations indicate that both the foraminiferal host and diatom symbionts are able to enter dormant states and recover once reintroduced to the light. This mutual dormancy could be why the acquisition of diatom symbionts was such a breakthrough in the evolution of larger benthic foraminifers, and how it contributed to their success.

Further research can address some of the questions this experiment has raised. An obvious first step would be to perform longer aphotic treatments to determine whether there is an upper bound to the ability of *Am. gibbosa* to recover. Similarly, extending this research to other taxa may help to understand how widespread aphotic dormancy may be as a survival strategy. Observing a variety of photosymbiotic foraminifers that host other symbiont taxa may help understand both the evolutionary origins of the strategy as well as the extent of its implications for assemblage interpretation. For instance, Smith & Hallock (1992) observed lower survival ability over shorter time scales in the chlorophyte-bearing miliolid *Archaias angulatus*; Lee et al. (1979) observed higher nutritional requirements for chlorophyte symbionts, which could play a role in determining the viable length of aphotic dormancy.

Another question is, what occurs between 12 and 15 months to cause the observed decrease in recovery rate? If, as is indicated by the ANCOVA results, the rate of recovery changes significantly between the 12- and 15-month treatments (Fig. 4.1), filling in the gaps in treatment length may reveal how and why recovery ability decreases over time. The use of a wider range of light levels post-treatment could also help understand the conditions required for recovery in the field.

Other approaches may inform what happens between 12 and 15 months, and possibly beyond, that reduces the ability of the foraminifers to recover. Proteomic analysis may indicate whether there is a change in protein expression between the earlier and later treatments that may be related to survival and recovery. Similarly, the use of techniques to directly observe metabolism, such as ATP assays, could indicate whether there is a change to metabolism in that time range, as well as observing what is happening metabolically during recovery. Observations of the ultrastructure of the cell, for instance via Transmission Electron Microscopy or thin

section light microscope histology, could elucidate the intracellular responses of the host and symbiont across time in the aphotic treatments (TEM analysis of ultrastructure was unsuccessfully attempted in the course of this research; see Appendix III for details). Evidence of apoptosis or digestion of symbionts could indicate destructive cellular functions that could, over time, limit the ability of the foraminifers to recover.

Conclusions

This experiment revealed that the *A. gibbosa* holobiont is capable of surviving up to 12 months in total darkness, with 100% recovery of the sample population when reintroduced to normal light levels, and that ~80% recovery is possible following up to 20 months in darkness. Documenting the potential for recovery from extended periods of dormancy in the *A. gibbosa* holobiont has implications for interpretations of foraminiferal ecology, assemblage recovery post-disturbance, and assemblage interpretation. Further research is needed to determine how widespread this ability is amongst symbiont-bearing foraminifers, as well as the limits of recovery and the mechanics that affect survival and recovery ability.

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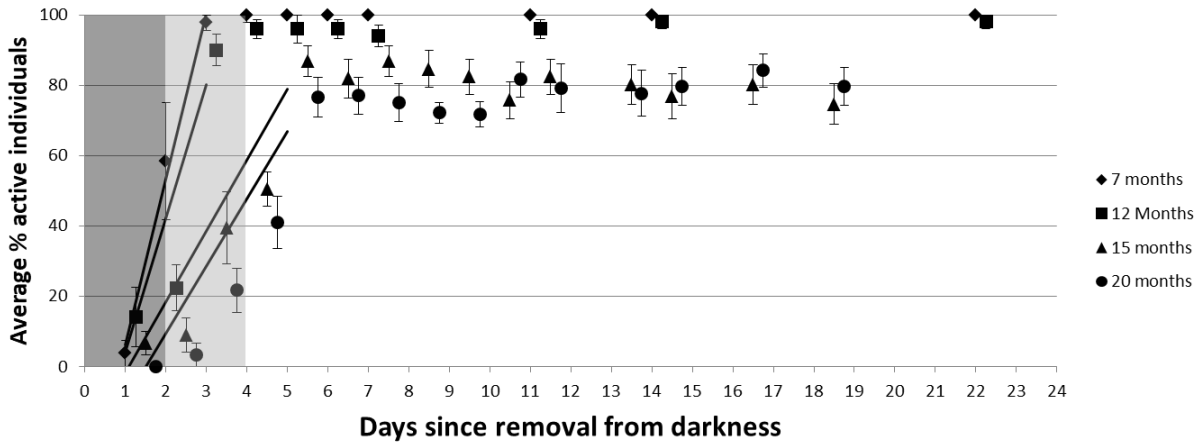

Figure 4.1: Average % active individuals per treatment per days of recovery. Treatments are offset within days for readability. Regression lines represent slope of the recovery portion of the figure for each treatment. The darker grey shading represents two days of heavy shading; the lighter grey represents two days of light shading; the rest of the figure represents recovery in full ambient light. Error bars represent standard error.

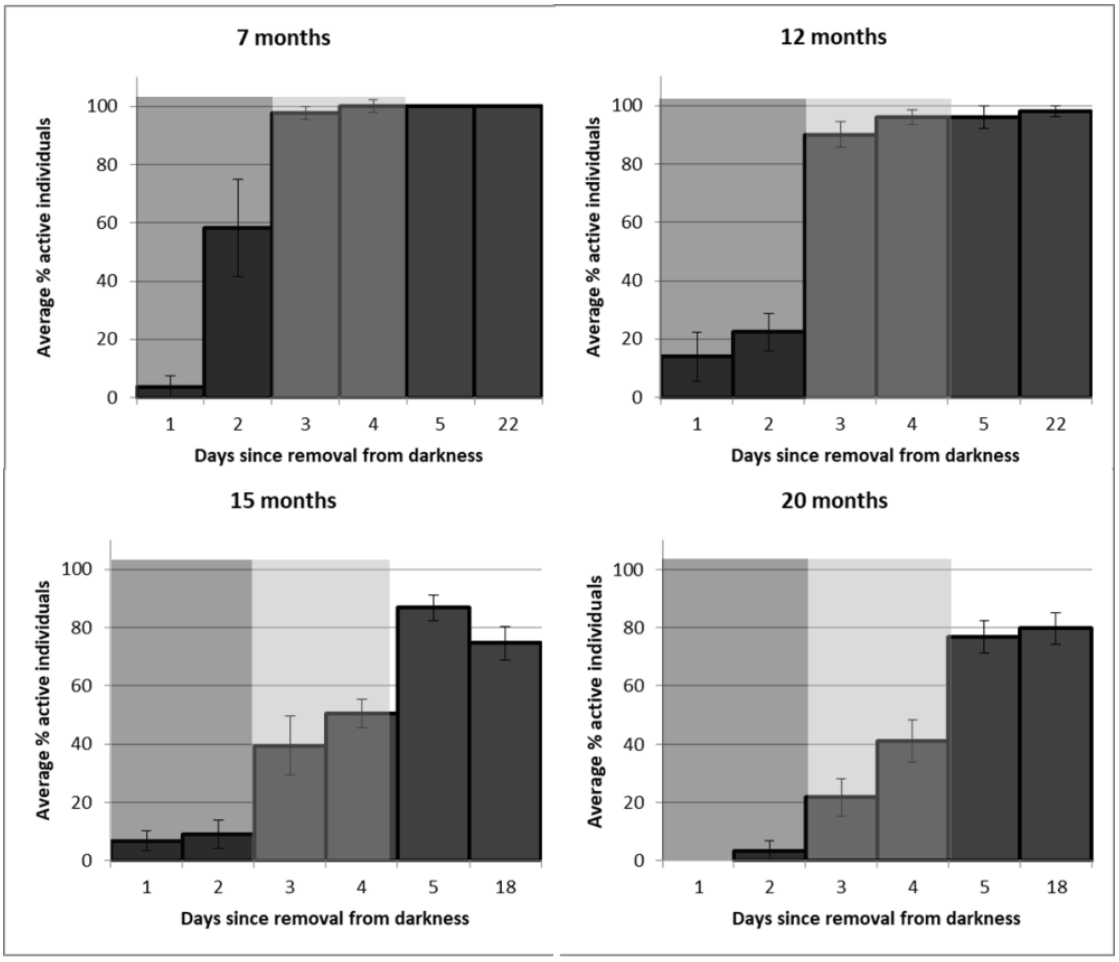

Figure 4.2: Average % active individuals per day across initial recovery, compared to sampling approximately two weeks later. Histograms show an initial rapid recovery, followed by consistent asymptote. Days needed for recovery differed between the 7- and12-month pair and 15- and 20-month pair. Grey shading represents amount of shade given to recovering foraminifers; dark grey is heavy shading, light grey light shading, no grey is full ambient light. Error bars represent standard error.

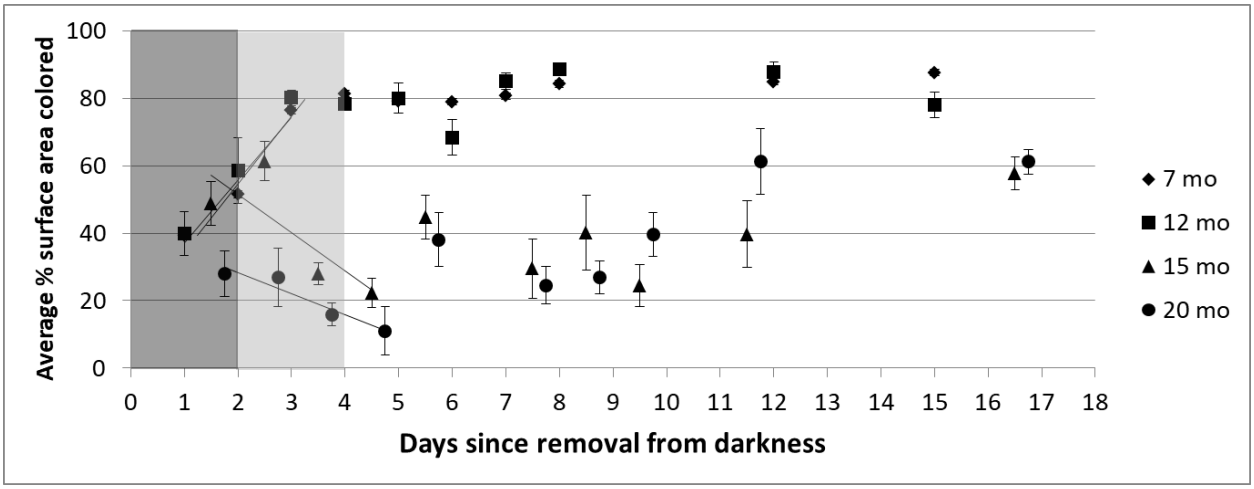

Figure 4.3: Average % surface area colored per treatment per day of recovery. Treatments are offset within days for readability. Regression lines represent slope of the recovery portion of the figure for each treatment. For the 15- and 20-month treatments, the final day (16) was taken from the full treatments that had not been photographed daily. The darker grey shading represents two days of heavy shading; the lighter grey represents two days of light shading; the rest of the figure represents recovery in full ambient light. Error bars represent standard error.

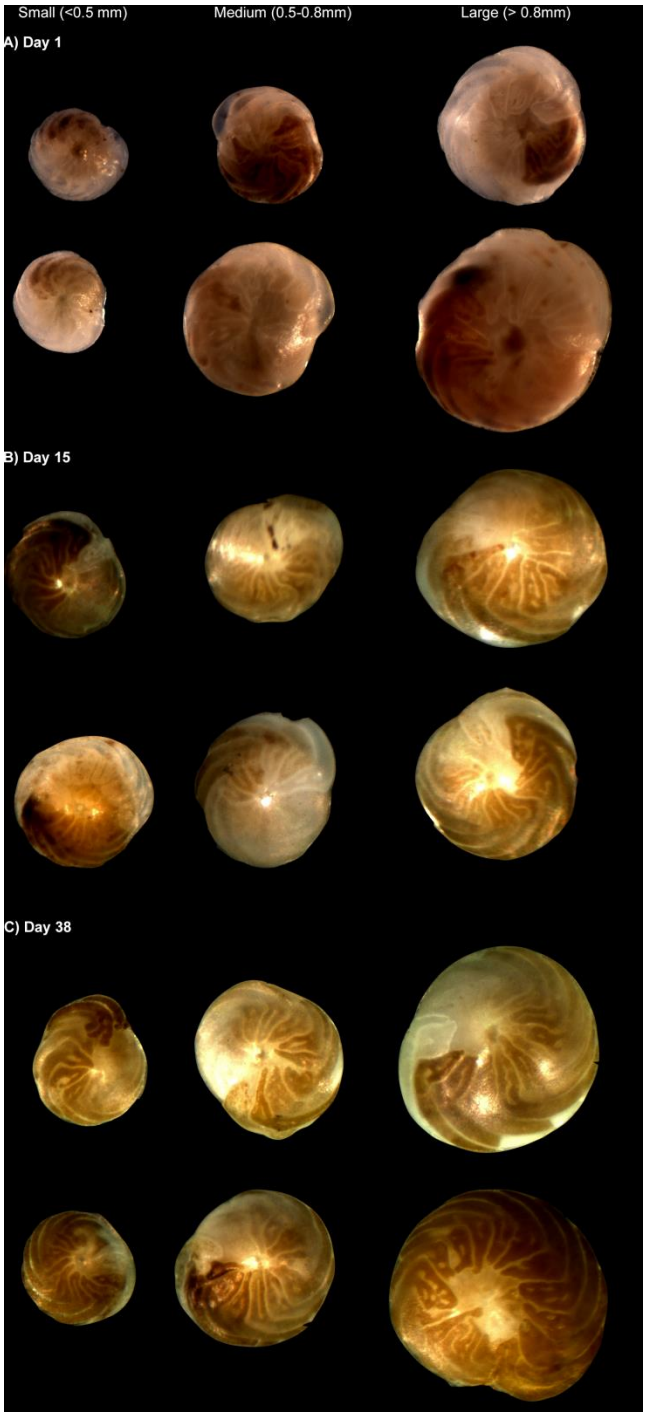

Figure 4.4: Recovery of color in *Amphistegina gibbosa* held in darkness for 15 months**:** Recovery of color in small, medium and large individuals held in darkness for 15 months on A) Day 1, B) Day 15 and C) Day 38 after return to lighted conditions.

Table 4.1. Results of statistical comparisons of response variable by treatments: summary of the pairwise p-values of statistical differences between the entire activity recovery curve over time (distribution), the slope of linear portion of the activity recovery curve (slope), and the Percent Surface Area Colored (PSA) recovery curve over time. Bolded entries indicate a significant difference at a p-value of 0.05.

	12 months	15 months	20 months
7 months	Distribution: p=0.07 Slope: p=0.48 PSA: p=0.6	Distribution: p=0.06 **Slope: p=0.001** **PSA: p=0.003**	**Distribution: p=0.02** **Slope: p=0.001** **PSA: p=0.001**
12 months		Distribution: p=0.3 **Slope: p=0.002** **PSA: p=0.004**	Distribution: p=0.14 **Slope: p=0.001** **PSA: p=0.002**
15 months			Distribution: p=0.6 Slope: p=0.92 **PSA: p=0.02**

CHAPTER FIVE

RECOGNIZING PATTERNS IN THE ACTIVITY AND COLOR RECOVERY OF *AMPHISTEGINA GIBBOSA* FOLLOWING REMOVAL FROM APHOTIC CONDITIONS

Abstract

Dormancy and subsequent recovery has been observed in the diatom symbiont-bearing foraminifer *Amphistegina gibbosa* following extended periods of darkness. Survival and recovery of symbiont population (indicated by a recovery of color in the foraminifers) was observed following up to 20 months in darkness (see Chapter 4). Here, the data from that study is further analyzed, identifying the presence of a gradient of survival and color recovery over time. In addition, the computer-aided image-analysis techniques used in Chapter 4 are compared to a qualitative ranking, revealing that the two measures correlate well, but that the image-analysis methods may identify signals missed by the lower resolution qualitative ranking. Qualitative comparisons of color hue were performed, indicating, that although specimens from the different length treatments varied in health upon removal, they all tended towards healthy coloration over time. Analyses of these color recovery measures comparing repeatedly photographed and non-photographed replicates, however, also indicated that sensitivity of the holobiont to photic stress increased as the time held in total darkness increased.

Introduction

As discussed in the previous chapters, dormancy and associated triggers are becoming more widely recognized in the Foraminifera (see Ross & Hallock, 2016, and references therein). As presented in Chapter 4, one of these triggers, at least for photosymbiotic foraminifers, is darkness. Lee et al. (1991) found that *Amphisorus hemprichii* Ehrenberg (a dinoflagellate-bearing miliolid) and *Amphistegina lobifera* Larsen (a diatom-bearing rotaliid) survived for 8 and 13 weeks in darkness, respectively. Smith & Hallock (1992) reported on the survival of *Archaias angulatus* (Fichtel & Moll) (a chlorophyte-bearing miliolid) and *Amphistegina gibbosa* (d'Orbigny, 1826) (a diatom-bearing rotaliid) held in total darkness, observing that some individuals survived up to 3 and 12 months in darkness, respectively. The surviving *A. gibbosa* subsequently regained normal symbiont color and behavior after being returned to a normal day/night light cycle. The experiments presented in Chapter 4 demonstrated recovery of *A. gibbosa* held in darkness for up to 20 months, as well as quantifying the rate and extent of the recovery of symbiont-derived color as a percent of shell-surface area showing symbiont coloration over time after removal from darkness (results and response parameters summarized in Table 5.1).

The results reported in Chapter 4 raised a number of questions that were not fully addressed due to space limitations in the manuscript submitted for publication. Fortunately, additional data analysis allowed some of these issues to be further explored. The first issue was the differences between the recovery of foraminifers held in darkness for 7 and 12 months versus those held for 15 and 20 months, with more limited evidence for a gradient of recovery. The

second topic for elaboration was a comparison of previously-used ranking approaches to the quantitative approach using image analysis. The final topic was whether additional analysis of the experimental data could promote better understanding of the apparently extreme photic sensitivity of *Amphistegina gibbosa*.

Materials and Methods

Basic Methods

The goals of Chapter 4 were to quantify both survival of *A. gibbosa* following extended aphotic conditions of 7, 12, 15 and 20 months, as well as the recovery of symbiont color following removal from these conditions. Individual *A. gibbosa* were picked under a stereomicroscope from reef rubble collected from 18 m water depth at Tennessee Reef in the Florida Keys (24.7523°, –80.7549°). Groups of five individuals were placed in microcentrifuge tubes (1.5 ml) with pierced holes at the top and bottom to allow for water and gas exchange. These tubes were then placed in sealed, semi-opaque screw-top jars, filled to the top with seawater collected at Tennessee Reef; the jars were wrapped in two layers of aluminum foil and placed in an incubator at a constant temperature of 25°C. Individuals were held in complete darkness and sampled at 7, 12, 15, and 20 months. Two sets of experiments were established five months apart, allowing 7- and 12-month treatments to be sampled on the same day, as were the 15- and 20-month treatments.

When removed from the treatments, replicates were moved to well plates in a semi-dark room (ambient light < 1µM photons m^{-2} s^{-2}, measured with a LI-COR photometer); when not being directly handled, specimens were kept under a light proof box, in which the photometer indicated no presence of light, even in a fully lit room. The 7- and 12-month treatments were left

to recover on a lab bench, near a window; laboratory temperature was consistently ~25°C, and light levels varied from < 1µM photons m^{-2} s^{-2} at night with the laboratory lights off, to ~ 7 µM photons m^{-2} s^{-2} during the afternoon when light directly entered the windows and the overhead lights were on. For the 15- and 20-month treatments, technical issues led the laboratory to be ~17°C, which is below the healthy temperature range for *A. gibbosa*. These samples were allowed to recover in an incubator with ambient light levels (depending on positioning, which was rotated after daily sampling) of ~3–7 µM photons m^{-2} s^{-2} on a 12 hour light/dark cycle at 25°C; as discussed in Chapter 4, recovery conditions for all treatments were largely equivalent.

Separate replicates were removed for daily photography of color recovery over time post-treatment. For the 7- and 12-month treatments, these consisted of three replicate wells of five individuals; for the 15- and 20-month treatments, a lack of specimens by the end of the treatment due to subsampling for other experiments and trials necessitated the use of only a single replicate of five individuals. These replicates were photographed as close to daily as possible for at least seven days; this led to exposure to light exposure of up to ~20 µM photons m^{-2} s^{-2} for up to five minutes during photography.

To minimize possible photic stress after being held in the dark for such long periods, the samples were initially covered in a double layer of light-attenuating mesh for two days, which decreased light levels by 80%; a single layer of mesh for another two days, which decreased light levels by 40%; and then a return to full ambient light levels, either on the lab bench or within the incubator. Observations of activity (extruded reticulopodia, attachment to side of well plates, presence of waste material) were made every day, as close to 24 hours following the previous observation as possible; these measurements of activity were used as a visual indicator of life, and of recovery from dormancy. Water in all well plates was changed every 48 hours.

Bimodal or Gradient Response?

The first issue to be further evaluated was the difference in response parameters between the foraminifers held in darkness for 7 and 12 months versus those held for 15 and 20 months, with more limited evidence for a gradient of recovery. As presented in Chapter 4, using activity as a proxy for recovery, comparisons among the treatments using a 2-way Repeated Measures ANOVA with no replication revealed significant differences among the treatments, with pairwise testing indicating differences only between results of the 7- and 20-month treatments. This result contrasted with the results of a non-parametric ANCOVA analysis of the linear recovery-slopes, in which the 7- and 12-month treatments were not significantly different from each other, but were significantly different from the 15- and 20-month treatments (results summarized in Table 5.2). To further assess whether the resulting post-recovery distributions were significantly different, this chapter separated the linear recovery slopes and asymptotic plateaus of the recovery patterns, and compared the asymptotes across treatments. In addition, a Canonical Analysis of Principle Components (CAP) was performed to provide an alternate visualization of dissimilarity among treatments.

Is Image Analysis Worth the Effort?

In Chapter 4, ImageJ image-analysis tools were used to calculate the percent of foraminiferal surface area exhibiting symbiont color over time (percent surface-area colored). In addition, qualitative rankings of colored surface area (color-coverage ranking), similar to the "bleaching rank" used in studies of *A. gibbosa* experiencing photic stress (e.g., Williams et al., 1997), and of color hue (color rank) (terms summarized in Table 1), were made. A color-

coverage ranking of 1 indicated a healthy, fully colored individual; 2 indicated ~75% of the surface-area colored; 3, ~75%- 25% colored; 4,<~25% colored; 5, no visible color; and 6, dead. A color ranking of 1 indicated color lighter than that seen in a normal, healthy individual; 2, healthy color; and 3, darker than healthy color, often associated with acute stress or photic damage (e.g., Williams, 2002). This chapter presents these data, as well as presenting correlations between the methods. In addition, a CAP was performed using the combination of all three measures to provide an alternate visualization of similarities among treatments.

How Sensitive are **A. gibbosa** *to Photic Stress?*

To record color recovery over time, two sets of replicates from each treatment were compared. The photographic replicates were photographed daily for at least seven days starting from the initial removal from darkness. In the replicates visually observed daily for signs of activity, color recovery was not regularly recorded and no photographs were taken until the end of the observation period. Especially for specimens kept in the dark for 15 and 20 months, I hypothesized that exposure to higher light intensities during daily photographs (~20 µM photons m^{-2} s^{-2} for up to 5 minutes) may have induced photic damage, resulting in less recovery of normal color than in specimens that were primarily exposed to ambient light in the laboratory or incubator (~7 µM photons m^{-2} s^{-2}) (Fig. 5.1). To test this hypothesis, specimens that had been photographed daily and those only visually evaluated daily, then photographed at the end of the observation period, were compared across treatments using the three color-recovery response parameters.

Statistical Methods

All statistics were calculated using either MATLAB with the Fathom toolbox (Jones, 2015) or Microsoft Excel with the Real Statistics Resource Pack software (Release 5.4) [Copyright (2013 – 2018) Charles Zaiontz. www.real-statistics.com]. For activity and color recovery over time, distributions were compared using a non-parametric, 2-way Repeated Measures ANOVA to account for the repeated sampling of the population [f_NPManova (Jones, 2015)](Sokal & Rohlf, 1981). Daily averages were used for these analyses. To determine correlations between the different measures of color recovery (percent surface-area colored, color-coverage ranking, color ranking), Spearman's correlation coefficients and associated p-values were calculated in MATLAB. To compare the response parameters for specimens photographed daily (for 15 days in the 7- and 12-month treatments, and 11 days for the 15-and 20-month treatments) to response parameters for specimens only photographed at the end of the recovery observations (i.e., after 15 days for 7- and 12-month treatments and 16 days for 15- and 20-month treatments days), Mann-Whitney tests were used. To compare the response parameters among treatments for specimens only photographed at the end of the post-darkness observation period, a non-parametric one way ANOVA test [f_Permanova (Jones, 2015)] was used.

Results

Bimodal or Gradient Response?

Further analysis of the response parameters not only supported significant differences between the 7- and 12-month treatments compared to the 15- and 20-month treatments, but also supported the hypothesis of response gradients (Table 5.1). As reported in Chapter 4, a 2-factor ANOVA with no replication using the daily averages from all of the treatments indicated a

significant difference between the mortality recovery distributions (factors 1 and 2, $p = 1x10^{-3}$). Pairwise comparisons of just the asymptotic portions between the treatments sampled on the same day indicated no significant differences between 7- and 12-month treatments and the 15- and 20- month treatments, but significant differences between all other treatments (see Fig.5.2); pairwise p-values are provided in Table 5.2.

A Canonical Analysis of Principal Components was performed to visualize similarities in activity recovery among treatments (Fig. 5.3A). Separation along axes represents differences among replicates of all treatments; Axis 1 represents 92% of observed difference, and Axis 2 represents 8%. The 7- and 12-month replicates visibly clustered together; the 20-month replicate well separated and more similar to the 15-month replicates than either the 7 or 12 month replicates. Thus, the 15 month replicates appear to be intermediate between the two extremes.

Is Image Analysis Worth the Effort?

Differences in recovery among treatments

As presented in Chapter 4, comparisons between recovery of the percent surface-area colored over time (Fig. 5.1A) at a p-value of 0.05 did not show a significant difference between 7- and 12-month treatments, but did show differences between all other treatment pairs (Table 5.3). Comparisons between the color-coverage ranking of the different treatments indicated significant differences among treatments (factors 1 and 2, p-values = $1x10^{-3}$) at a p-value of 0.05, and pairwise comparisons indicated significant between all treatments (Table 5.3). Similarly, comparisons between the color ranking indicated significant differences between all treatment pairs (factors 1 and 2, p-values = $1x10^{-3}$) at a p-value of 0.05 (Table 5.3).

Color Recovery Correlation

The three response parameters assessing color recovery following extended dormancy were compared. Correlations were calculated between the measurements of percent surface-area colored and both color-coverage ranking and color ranking for each treatment (Table 5.4). For the 7-, 12- and 20-month treatments, significant negative correlations were found between the percent surface-area colored and color-coverage ranking, and no significant correlation was found for the 15-month treatment. For the percent surface-area colored and color ranking, a significant correlation coefficient was only found in the 12-month treatment.

A Canonical Analysis of Principal Components was performed to visualize similarities among treatments based upon color recovery (Fig. 5.3B). Separation along axes represented differences among replicates; Axis 1 represented 47% of observed difference, and Axis 2 represented 52%. Replicates from the 7- and 12-month treatments visibly clustered together; replicates from the 15- and 20-month treatments were well separated from 7- and 12-month replicates on Axis 1, and separated from each other along Axis 2.

How Sensitive are A. gibbosa to Photic Stress?

Mann-Whitney tests comparing the final day that specimens were photographed daily (day 8 for all treatments) with the first day of photography for visually observed treatments (day 15 for the 7- and 12-month treatments; day 16 for the 15- and 20-month treatments)(Fig. 5.1) showed a significant difference at a p-value of 0.05 for percent surface-area colored for 12 months, but not for other treatments; no differences in color-coverage ranking; and no differences in color ranking; p-values are provided in Table 5.5.

Non-parametric one way ANOVA comparison of percent surface-area colored for the originally visually observed treatments showed a significant difference among treatments ($p = 0.001$). Follow up pairwise comparisons between treatments showed no significant differences between the 7- and 12-month treatments or the 15- and 20-month treatments but significant differences between all other comparisons (see Table 5.6 for pairwise p-values). Comparison between the color-coverage ranking showed a significant difference between treatments ($p = 0.02$). Follow up pairwise comparisons showed no significant differences between 7 and 12 months, 12 and 20 months, or 15 and 20 months, but significant differences between 7 and 15 months, 7 and 20 months, and 12 and 15 months. Pairwise comparisons of color rankings showed significant differences between all treatments (see Table 5.6 for pairwise p-values).

Discussion

Bimodal or Gradient Response?

In Chapter 4, comparisons of the activity-recovery slopes revealed no significant difference between the 7- and 12-months treatments, or between the 15- and 20-month treatments, but significant differences between all other pairs, indicating a bimodal response. Further analyses presented in this chapter comparing the asymptotic-plateau portions of the recovery distributions showed no significant differences between the 7- and 12-month treatments, and the 15- and 20- month treatments, again indicating a bimodal tendency (summarized in Tables 5.1 and 5.2).

Despite this evidence, comparisons of the full activity recovery distributions in Chapter 4 showed significant differences only between the 7- and 20-month treatments, indicating a gradient of decline in recovery over time. Thus, although the rates of recovery and the

asymptotic plateaus of recovery exhibited bimodality, the combination of rate of recovery (slope) and extent of recovery (asymptote) resulted in a gradient of response, despite the visual separation between the 7- and 12-month and 15- and 20-month treatments seen in Fig. 5.2. This agrees with the pattern visible in the canonical analysis of activity recovery, where the 15-month treatment appeared to be intermediate between the more defined 7- and 12-month treatment cluster and the 20-month treatment (Fig. 5.3A).

These results are also interesting in light of findings by Ross & Hallock (2018) that *A. gibbosa* removed from the darkness after 62 days show some level of metabolic recovery (indicated by the use of the fluorescent probe CellTracker Green CFMDA [CTG]) within 30 minutes, with normal symbiont color returning within 99 hours (see Figure 5 in Chapter 3), while the same level of recovery took significantly more time following the longer 7–20 month treatments, and sometimes never recovered to the same extent (see Figure 4.4). Using CTG following longer treatments, such as those in this chapter, may illuminate differences in the timing of metabolic increase following return to light, as well as the latency between the beginning of metabolic recovery, and activity, and symbiont recovery.

Is image analysis worth the effort?

*Comparison of color-coverage measures***:**

As reported in Chapter 4, the percent surface-area colored did not show a significant difference between the 7- and 12-month treatments, but did show significant differences between all other treatments (Fig. 5.1A; Table 5.3). The color-coverage ranking showed a similar pattern, except for the indication of a difference between 7- and 12-month treatments (Fig 5.1B; Table 5.3). In addition, these two measures were found to be significantly negatively correlated

strongly in all treatments, except for 15 months (Table 5.4); the negative correlation is the consequence of the inverse ranking in which the highest amount of surface-area colored in the color-coverage ranking was ranked 1, and the least surface-area colored ranked 5. Thus the color-coverage ranking and percent surface-area colored appear to be measuring the same response.

However, the indication of a significant difference between the color-coverage ranking between the 7- and 12-month treatments in the daily photographic record, where the percent surface-area colored showed none, and the lack of a significant difference between the 12- and 20-month treatments in the non-daily photographic record, despite the correlation, indicates that the measures are not fully equivalent. While some details may be missed by the relative coarseness of the ranking measure, as well as the inherent bias involved in qualitative measurements, the differences observed in the color-coverage ranking show more evidence for a gradient of response than the differences in percent surface-area colored (Table 5.1), consistent with the activity-recovery measures. In addition, image analysis is more labor intensive, and requires exposure to damaging light levels. This highlights the situational utility and usefulness of both qualitative ranking systems and more quantitative computer-aided image analysis in examining changes in color in foraminifers that host algal endosymbionts.

Color Ranking:

Compared to the color-coverage ranking, the color ranking (Fig. 5.1C) was not useful as a measure of recovery, though it did indicate differences in the initial coloration of the specimens when removed from their treatments. Over time, all of the treatments tended to approach normal coloration (a color ranking of two).

Color ranking (Fig. 5.1C) was also only weakly correlated to the percent surface-area colored in the 12 month treatment, and not significantly correlated in other treatments (Table 5.4). This may be because lighter than normal color is likely an indication of how deep the visible color is within the shell. In larger individuals, especially, initial symbiont recovery may be occurring deep within the shell, filling out from the middle (e.g., Fig. 4.4). Because the technique examined the surface area of the foraminifers as if they were flat, as opposed to their true lenticular shape, volume was not considered in any measurements; color originating from deeper within the shell would appear lighter under microscope conditions. Darker than normal color, on the other hand, can indicate photooxidative stress (e.g., Talge & Hallock, 2003), but does not seem to be strongly related to the amount of surface coloration visible.

The canonical analysis using all three measures of color recovery (Fig. 5.2B), indicated strong similarity between the 7- and 12-month treatments, which were more different from the 15- and 20-month treatments, which in turn were relatively different from each other. This is in contrast with the canonical analysis based upon activity recovery (Fig. 5.3A), which showed high similarity between the 7- and 12-month and between 15- and 20-month treatments. Although that analysis indicated that 15 months could be intermediate in terms of activity recovery, the analysis of color recovery does not indicate such a relationship, instead showing a bimodal distribution. This contrast implies that the relationship between treatment time and recovery ability differs somewhat in the foraminiferal hosts, as indicated by activity recovery, than in the symbiont population, as indicated by color recovery, an observation consistent with the results reported by Smith & Hallock (1992).

How Sensitive are A. gibbosa *to Photic Stress?*

Comparisons of surface-area colored on the final day of photography of the visual-observation replicates support the interpretation of the color recovery canonical analysis, as the 7- and 12-month treatments were not significantly different, and the 15- and 20-month treatments were not significantly different, while other pairs were significantly different. This is consistent with results from the asymptotic portions of the activity-recovery curves, which also revealed bimodality in recovery responses. This match between the extent of foraminiferal activity recovery and extent of color recovery is in contrast to the findings of Smith & Hallock (1992), who found that the symbionts tended to outlive the foraminiferal hosts, leading to color recovery with no associated recovery of foraminiferal activity,

This is also in contrast to the patterns seen in Fig. 5.1A-B, where the percent surface-area colored actually seems to decrease and the color-coverage ranking to increase over time in the 15- and 20-month daily photographic treatments, indicating that coloration became less healthy over time following removal from darkness. This, combined with the color recovery CAP (Fig. 5.3B), which shows separation between the 7- and 12-month treatments, the 15-month treatment, and the 20-month treatment , supports the hypothesis that the symbiont populations became more sensitive to light stress the longer the foraminifers are kept in the dark.

If this is accurate, comparisons of the final day of photography of the daily photographic record (repeatedly exposed to elevated light levels) with the first day of photography of the non-daily photographic record (largely kept out of elevated light levels) would hypothetically show a significant difference; the days from the replicates that were not photographed daily (the uncolored marks in Fig. 5.1A-B) appear to show elevated levels of percent surface-area colored and color-coverage ranking compared to the rest of the distribution. This may be because they

are from the full treatment, which was not photographed daily during recovery. However, comparisons showed no significant differences between measures of color between these days, except for the percent surface-area colored in the 12 month treatment. This comparison may be complicated by the small sample size of the 15 and 20 month daily photographic record (5 individuals), compared to the size of the non-daily photographic record (39 and 49 individuals, respectively).

As summarized in Table 5, however, comparing the final day of photography for the non-daily photographic record shows a bimodal distribution of percent surface-area colored, with the 7- and 12-month treatments and the 15- and 20-month treatments pairing. These pairs also occur in the color-coverage ranking, although complicated by the fact that 12 months is not significantly different from 20 months (summarized in Table 5.6). This suggests a stronger separation between the pairs of treatments when not exposed to elevated light during daily photography. This could explain why the 15- and 20-month daily photographic record are significantly different from each other, while the non-daily photographic record are not; the effects of naturally occurring differences are overwhelmed by those caused through exposure to elevated light levels. Figure 5 in Chapter 3 shows the extent of color recovery in individuals kept in the dark for 62 days under both light and fluorescence microscopy. Despite exposure to high energy light associated with the fluorescence microscope, these specimens exhibited fewer obvious signs of photic stress (e.g., mottling or abnormally dark coloration) than observed in the treatments discussed in this chapter. This provides further support for the hypothesis that light sensitivity increases as the length of time in the dark increases.

Bimodal vs. Gradient: Ecological and Evolutionary Implications

Table 5.1 summarizes the differences between the response variables discussed in chapters 4 and 5. Within these data there is evidence for both bimodal and gradual change in the survival and recovery ability of *A. gibbosa* after removal from long term aphotic conditions. The percent of surface area colored in the treatments that were not photographed until the end of the recovery period (non-photographic record treatments, or NPR in Table 1) and the asymptotic portion of the activity recovery curve both show bimodal separation between treatments, with the 7- and 12-month treatments and 15- and 20-month treatments not significantly different from each other, but different from the treatments in the other pair. These response variables were also the only ones to take into account the longest term observations, up until the end of the observation period. This could suggest that, over time, differences in recovery ability post-removal lead to a bimodal distribution that was not present earlier in recovery. This interpretation is confused by the results of the color coverage-ranking in the non-photographic treatments, which do not show a clear bimodal or gradual recovery pattern with increasing treatment length.

The differences in the percent surface area covered in the daily photographic replicate (PR) and the linear portion of the activity recovery curve show a partial gradient of recovery, with the7- and 12-month treatments not differing significantly, but being different from the 15-month and 20-month treatment, which are also different from each other. This could suggest that survival and recovery abilities start to decrease after a certain period of time in darkness, possibly between 12 and 15 months, after which, the longer the treatment, the lower the ability to recover. Both of these measures include the earliest observations made, and could mean that the survival ability of the intermediate 15-month treatment was initially higher than that of the 20-

month treatment, becoming more similar over time. This pattern can be seen, for instance, in the activity recovery curves of Figure 5.2, or in the CAP plots in Figure 5.3.

The color coverage-ranking of the daily photographic treatment and the total activity recovery curve, on the other hand, both show evidence for a full gradient of recovery beginning with the 7-month treatment. The difference between the percent surface-area colored and color coverage-ranking results, with the former showing a limited gradient and the latter a full gradient, could reflect differences in the sensitivity of the methods.

These results also differ from those of Smith & Hallock (1992), who observed that *A. gibbosa* recovery ceased entirely between 12 and 18 months, although its diatom endosymbionts were able to recover up to 18 months. As discussed in Chapter 4, there are a number of possibilities for the differences in observed recovery, such as light exposure regime upon removal from darkness and extent of time given for recovery. As such, it is difficult to tell whether the results of Smith & Hallock (1992) provide evidence for a bimodal or gradual change in survival and recovery ability. It is possible that the cessation of recovery between 12 and 18 months reflects the same decrease in survival and recovery ability seen in the hypothetical bimodal distribution, with the effects exacerbated by the differences in experimental procedure, such as exposing the longer, apparently more photosensitive, treatments to full environmental light levels immediately after removal from darkness. On the other hand, differences in experimental procedure may have allowed for the identification of a survival gradient in these new experiments that was not apparent given the procedures used by Smith & Hallock (1992).

The ability to survive extended periods of darkness could be especially advantageous for a species such as *A. gibbosa*, which can live over a variety of depth and light levels. When living in marginal photic environments, disruptions such as increased turbidity in the wake of storms

could cause physiologically significant changes in the amount of available light. Similarly, *A. gibbosa*'s benthic lifestyle in relatively energetic environments puts individuals at risk of burial. The ability to survive relatively short periods of darkness is therefore a beneficial adaptation for the species, especially if dormancy functions on timescales short enough to act during regular nightly light limitation.

Moreover, conditions that contribute to loss of light can also be highly seasonal. For instance, turbidity and burial could both be increased as a consequence of winter storms, and, depending on latitude, the length of the day and the amount of available light can both change significantly depending on the time of year. Because of the seasonality of these effects, the ability to remain dormant for periods on the scale of 6–12 months could be a valuable adaptation, especially for individuals living near the limits of light tolerance.

Under these conditions, a gradient of survival ability could indicate a general ability to go dormant, surviving until the reduced metabolism consumes all of the available cellular resources. On the other hand, it is possible that a bimodal or partial gradient, where recovery ability is relatively constant to a certain point before decreasing significantly, could reflect the evolution of dormancy mechanisms specialized to survive light limitations on seasonal time scales. In that case, survival up to a year could be a valuable adaptation, with longer lengths of dormancy unlikely to be related to a regular seasonal change that could be expected to pass. Regardless of whether recovery ability is bimodal or gradual, darkness-induced dormancy is a valuable adaptation in light limited environments, and the ability to survive seasonal light reduction could play a role in range expansion in species limited by both light and temperature, such as *A. gibbosa*.

Dormancy could also have implications for the use of foraminifers as proxies for reconstructing past climates and environmental conditions. If dormant individuals are not adding to their shell, the isotopic signal may not be reflecting the environment they are living in while they are dormant. In cases where dormancy may be seasonal, this may lead to an isotopic signal that only reflects environmental conditions for part of the year. In the case of winter dormancy, for instance, the isotopic signatures may only reflect summer temperatures, and could be different than if the foraminifer was building its shell year-round.

Further Research

Use of cytological measures, including thin section microscopy and Transmission Electron Microscopy, could help determine whether color recovery is due to the movement or reproduction of symbionts, changes in concentration of cellular materials in internal chambers being moved to outer chambers, or a combination of effects (TEM analysis of ultrastructure was unsuccessfully attempted in the course of this research; see Appendix III for details). The use of symbiont cell counts and measures of chlorophyll would also help explain the symbiont population recovery. Cytological methods could also help determine whether areas that remain uncolored, even as an individual recovers its color to its fullest extent, is due to cellular damage, isolation of areas, or another explanation entirely.

Treatment lengths between 12 and 15 months may help explain why there seems to be such a consistent separation occurring between treatments before and after those months, and repeating the experiment, possibly with larger sample sizes, could help determine whether the pattern is consistent. The use of quantitative measures of metabolism and of other measures of activity, such as protein expression, could elucidate the factors controlling dormancy and

associated recovery, and help explain why recovery ability decreases as treatment length increases.

Conclusions

The results of Chapter 4 and 5 are summarized below.

1. *Amphistegina gibbosa* can survive extended darkness of up to 20 months.
2. The response variables showed evidence of a gradient of recovery:
    a. all assessed parameters showed significantly "better" recovery of specimens in the 7-month treatment compared to the 20-month treatment;
    b. specimens in the 7- and 12-month treatments did not differ significantly in 6 of the 9 response parameters;
    c. specimens in the 12- and 15-month treatments differed significantly in 8 of the 9 response parameters; and
    d. specimens in the 15- and 20-month treatments did not differ significantly in 4 of the 9 response parameters.
3. The qualitative measurement of color recovery (color-coverage ranking) and the quantitative measure (percent surface-area colored) of symbiont color showed similar trends, but were not fully equivalent:
    a. the results from the qualitative measurements showed clearer evidence for a gradient in response than the quantitative measures; and
    b. the qualitative measure of color hue (color ranking) showed a gradient of color upon removal depending on the length of the treatment, but over time the color in each treatment became more like that seen in a healthy, "normal" individual

4. *Amphistegina gibbosa* appeared to become more sensitive to photic stress as the length of time in darkness increased:
    a. differences in color recovery were observed between treatments; and
    b. a difference in the color coverage between the final days of the 15- and 20-month daily photographic treatments, which were regularly exposed to heightened levels of light, to the non-daily photographic treatments show a visible, but non-significant, increase in color coverage in the non-daily photographic treatments (non-significance was likely related to small sample sizes); and
    c. comparisons between all of the non-photographic treatments suggested a more bimodal distribution, indicating that photic sensitivity played a role in structuring the observed gradient in color recovery.

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Table 5.1: Terms and significance groups of color parameters for the daily photographic record treatments (PR) and non-daily photographic record treatments (NPR), and for different portions of the activity recovery curve. For each parameter, treatments with the same letters are not significantly different from one another, and groups with different letters are significantly different; e.g., for PR Percent Surface Area colored, 7- and 12-month treatments are not significantly different (group A), but they are significantly different than the 15-month treatment (group B), and the 20-month treatment (group C).

Parameter Name	7 months	12 months	15 months	20 months
PR Percent Surface Area colored	A	A	B	C
PR Color Coverage Ranking (CCR)	A	B	C	D
PR Color Ranking	A	B	C	D
NPR Percent Surface Area colored	A	A	B	B
NPR Color Coverage Ranking	A	A, B	C	B,C
NPR Color Ranking	A	B	C	D
Asymptotic portion of activity recovery curve	A	A	B	B
Total activity recovery curve (Chapter 4)	A	A,B, C	A,B, C	B, C
Linear portion of activity recovery curve (Chapter 4)	A	A	B	C

Table 5.2: Distribution/Slope/Asymptote mortality comparisons p-values of comparisons of differences between the full distribution, linear recovery slope, and post-recovery asymptote of activity recovery between treatments. Bolded entries indicate significance at p=0.05.

	7 month	12 month	15 month
12 month	Distribution: p=0.07 Slope: p=0.48 Asymptote: p=0.28		
15 month	Distribution: p=0.06 **Slope: p=0.001** **Asymptote: p=0.001**	Distribution: p=0.3 **Slope: p=0.002** **Asymptote: p=0.001**	
20 month	**Distribution: p=0.02** **Slope: p=0.001** **Asymptote: p=0.001**	Distribution: p=0.14 **Slope: p=0.001** **Asymptote: p=0.001**	Distribution: p=0.6 Slope: p=0.92 Asymptote: p=0.17

Table 5.3: Comparisons of color recovery among photographic treatments : p-values of comparisons of differences in percent surface area colored (PSA), color coverage ranking (CCR) and color ranking (CR) of the daily photographic replicates between treatments in the daily photographic treatments. Bolded entries indicate significance at p=0.05.

	7 month	12 month	15 month
12 month	PSA: p=0.6 **CCR: p=0.001** **CR: p=0.001**		
15 month	**PSA: p=0.003** **CCR: p=0.002** **CR: p=0.001**	**PSA: p=0.004** **CCR: p=0.016** **CR: p=0.02**	
20 month	**PSA: p=0.001** **CCR: p=0.002** **CR: p=0.001**	**PSA: p=0.002** **CCR: p=0.001** **CR: p=0.001**	**PSA: p=0.02** **CCR: p=0.003** **CR: p=0.002**

Table 5.4: Correlations between color recovery measures: p-values of correlations between the percent of surface area colored and both color coverage ranking and color ranking, with r-values for significant correlations. Bolded entries indicate significance at p=0.05

	7 months	12 months	15 months	20 months
Color coverage ranking	**p < 0.001 r = -0.5**	**p < 0.001 r = -0.5**	p = 0.09	**p < 0.001 r = -0.4**
Color ranking	p = 0.45	**p = 0.02 r = 0.2**	p = 0.5	P = 0.3

Table 5.5: Comparisons of final day of daily photographic record (PR) treatments and first day of non-photographic record treatments (NPR) following end of daily PR photography. Bolded entries indicate significance at p = 0.05.

	7 months	12 months	15 months	20 months
Percent Surface Area colored	p = 0.8	p = **0.02**	p = 0.27	p = 0.89
Color Coverage Ranking	p = 0.95	p = 0.72	p = 0.3	p = 0.46
Color Ranking	p = 0.22	p = 0.25	p = 0.96	p = 0.18

Table 5.6: Comparisons between final days of non-photographic record (NPR) treatments: p-values of comparisons of differences in percent surface area colored (PSA), color coverage ranking (CCR) and color ranking (CR) of the non-daily photographic replicates between treatments. Bolded entries indicate significance at p=0.05.

	7 month	12 month	15 month
12 month	PSA: p=0.67 CCR: p=0.48 **CR: p =0.001**		
15 month	**PSA : p<0.001** **CCR: p=0.01** **CR: p =0.001**	**PSA : p<0.001** **CCR: p=0.016** **CR: p=0.02**	
20 month	**PSA : p<0.02** **CCR: p=0.002** **CR: p =0.001**	**PSA : p<0.001** CCR: p=0.11 **CR: p = 0.01**	PSA p=0.79 CCR: p=0.68 **CR: p = 0.002**

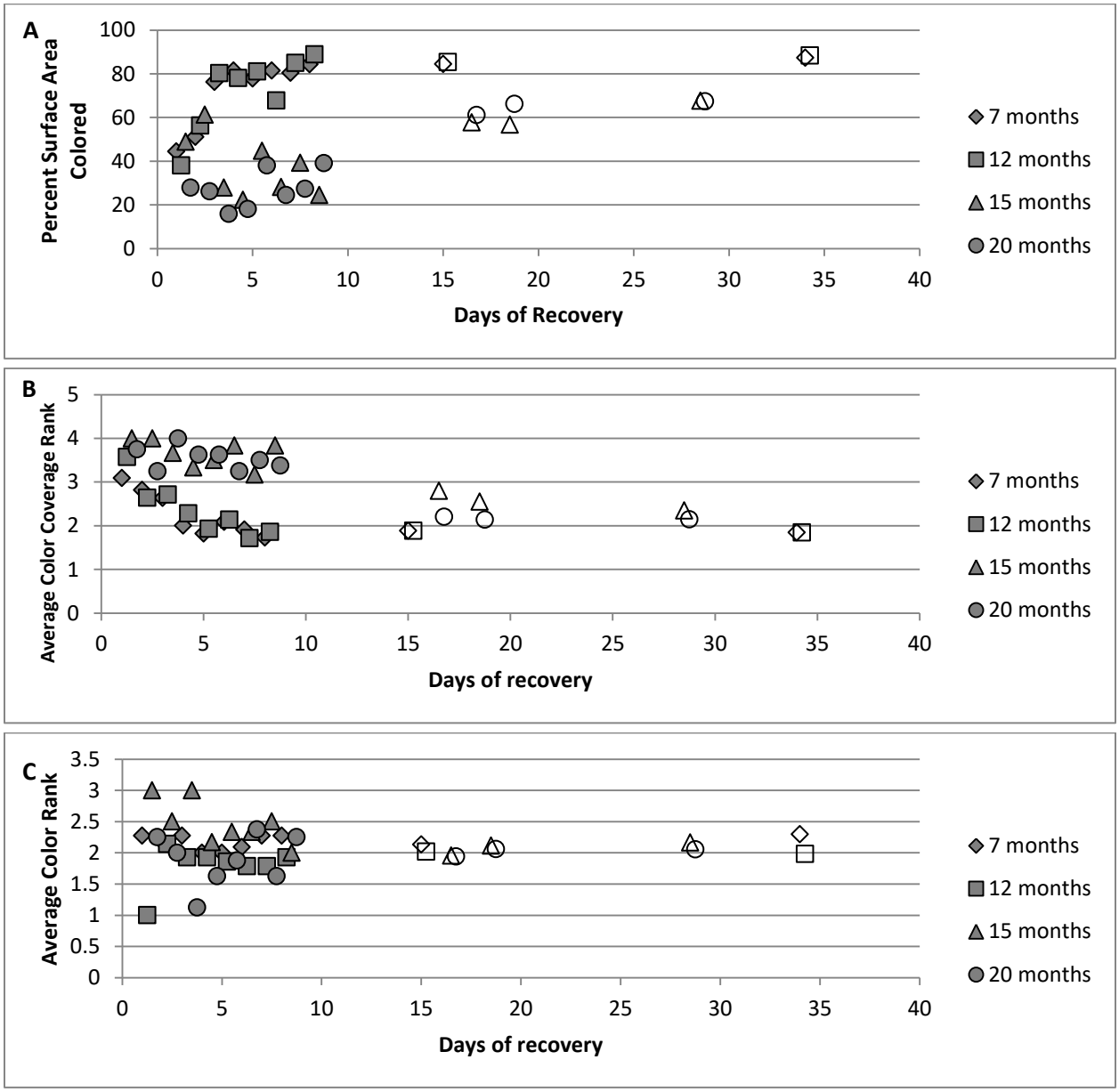


Figure 5.1: A)Percent Surface Area Colored, B) Color Coverage Rank, and C) Average Color Rank over time following removal from treatment. Unfilled points represent data from the non-daily photographic replicates.

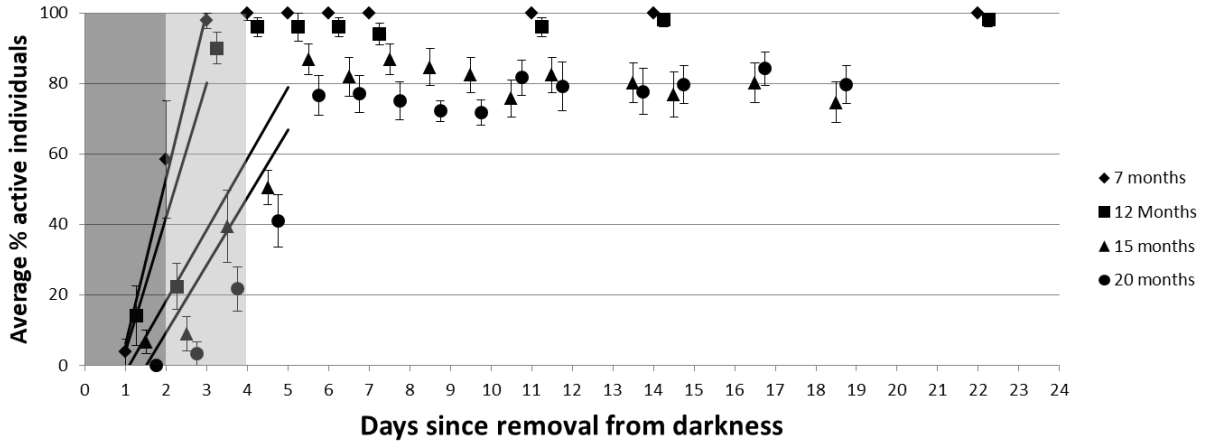
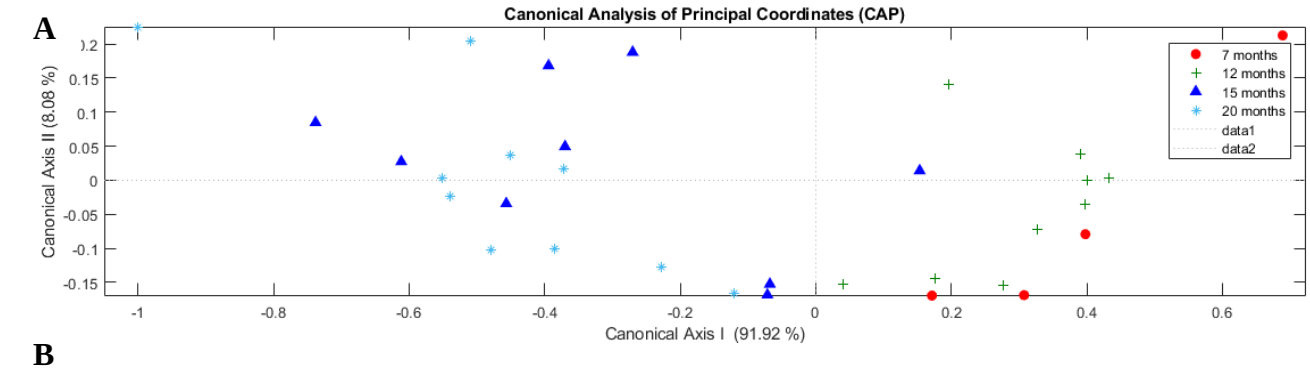


Figure 5.2: Average % active individuals per treatment per days of recovery. Treatments are offset within days for readability. Regression lines represent slope of the recovery portion of the figure for each treatment. The darker grey shading represents 2 days of heavy shading; the lighter grey represents 2 days of light shading; the rest of the figure represents recovery in full ambient light. Error bars represent standard error.



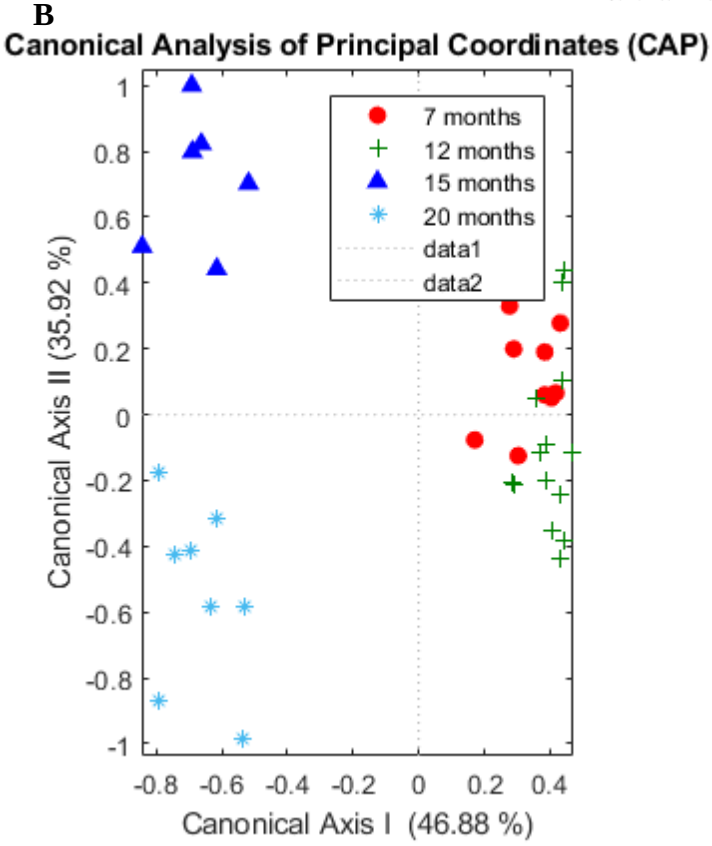


Figure 5.3: Canonical Analysis of Principal Coordinates (CAP) plots of differences in A): activity recovery over time and B): color recovery over time between treatments. Distance on axes represents differences between data points.

CHAPTER 6

CONCLUSIONS

Part I: Recent Literature

Since the initial publication of Chapter 1 (Ross & Hallock, 2016), the literature on foraminiferal dormancy has continued to grow, and the potential to enter dormancy has become more widely recognized as a potential driver of foraminiferal assemblage dynamics. For instance, Martinez-Colon et al. (2018) suggest that dormancy, in particular the presence of a cryptic propagule "seed bank" capable of rapid growth when conditions improve, could be contributing to relatively low incidences of foraminiferal test deformity in anthropogenically impacted portions of their Torecillas lagoon study site. Amao et al. (2018) suggest both propagule and post-propagule dormancy as strategies for foraminifers to survive the seasonally extreme conditions encountered in the Arabian Gulf.

More specifically, a number of studies concerning both the implications of cryptobiotic propagule dormancy and the effects of anoxia on foraminifers have underscored the role of dormancy in both dispersal and as a survival mechanism in the Foraminifera. Weinmann & Goldstein (2016) collected sediment from coastal Georgia and Florida and grew experimental assemblages from the fine (< 53 μm) sediment fraction under different salinity and temperature regimes. They found that these experimentally grown treatments contained many "exotic" species not found in the in situ assemblages, which has significant implications for understanding how assemblages may react to changing climate.

Weinmann & Goldstein (2017) found, using similar methods, that experimentally grown assemblages from shallow water sites in Georgia were dominated by species found offshore, instead of the species normally found in the marshy environments found landward of where the sediment was collected. They interpreted this as evidence that propagule transport from the terrestrial side of the gradient was limited, with propagules mainly originating offshore.
Both of these papers highlight the importance of understanding the role dormancy plays in the Foraminifera. They make it clear that propagule seed banks and patterns of propagule transport can play a role in the way foraminiferal assemblages will react to changing climate and sea level rise, as well as highlighting its importance in structuring assemblages in marginal environments in general.

Nomaki et al. (2016) used a combination of transmission electron microscopy (TEM) and nanometer-scale secondary ion mass spectrometry (NanoSIMS) to correlate ultrastructural features to concentration of ^{15}N and ^{34}S in *Ammonia* spp. incubated in both dysoxic and anoxic conditions with the addition of ^{15}N-labeled nitrate and ^{34}S-labeled sulfate. They found that ^{15}N and ^{34}S labeling was more apparent in the dysoxic treatments than the anoxic treatments, although still present under anoxia. One explanation is that the foraminifers were more active in dysoxic than anoxic conditions, consistent with dormancy as a survival mechanism in anoxic conditions. In addition, sulfur-rich electron dense bodies were identified, with significant ^{34}S-labeling under dysoxic conditions, although not under anoxic conditions. Nomaki et al. (2016) suggest that this indicates *Ammonia* spp. may synthesize sulfolipids through a sulfate-activation pathway, similar to that seen in *Entamoeba histolytica*, where it is crucial to encystment. They argue that, if a sulfate-activation pathway is involved with dormancy or encystment in *Ammonia*

spp., dormancy in the anoxia-incubated treatment could explain the lower incorporation of S in those individuals.

LeKieffre et al. (2017) used ^{13}C-enriched diatom biofilms to feed *Ammonia tepida* under both oxic and anoxic conditions, using correlated TEM and NanoSIMS imaging as well as bulk analysis of concentration and stable isotopic composition of total organic content and individual fatty acids to observe metabolic differences. They found that, under anoxia, there were changes in the ratios of different fatty acids, but not in the total amounts, indicating that some acids were being broken down in order to synthesize other acids, but no new fatty acids were being produced; this indicates the presence of very low levels of metabolic activity. They also found that neither total organic carbon (TOC) nor ^{13}C-enrichment increased after one day in anoxic conditions, consistent with visual observations that feeding on the labeled biofilm stopped entirely after the first day. However, the TOC did not decrease after Day 1, indicating minimal metabolic loss of carbon. LeKieffre et al. (2017) interpreted this pattern as indicative of a shutdown of aerobic metabolic processes under anoxia on a timescale of less than 24 hours. They also observed ultrastructural indicators of stress, such as an increase in lipid-drop abundance, in the anoxic treatment, suggesting a link between stressful conditions and onset of observed metabolic decrease. This change in lipid-drop abundance is also seen in *Ammonia becarii* exposed to Cu contamination (i.e., Le Cadre & Debenay, 2006); if these signs of stress are related to the onset of dormancy, then their presence in reaction to non-anoxia stress may suggest that dormancy is more widespread in *Ammonia* spp. than currently recognized.

Koho et al. (2018) observed cytosol thinning in *Ammonia* spp. isolated from anoxic sediments compared to those from oxygenated surficial sediments or oxic incubations. They suggest that this indicates dormant individuals consuming their own cytosol instead of actively

feeding, similar to the observations made by LeKieffre et al. (2017). This may allow *Ammonia* spp. found in deeper sediment layers to survive by intermittently respiring oxygen in micro-oxic sediment niches, for instance, as a result of bioturbation, and entering a dormant state when oxygen is unavailable. Interestingly, the observation that *Ammonia* spp. may be consuming their own cytosol to drive reduced metabolism under anoxic conditions is similar to that made in Chapters 4-5 of this dissertation that even *Amphistegina gibbosa* species exhibiting recovery of healthy color after removal from darkness often exhibited some level of "mottling", or presence of white spots. These spots could indicate portions of the cell that had been degraded during the time that the individual was in darkness, in order to maintain necessary metabolic activity.

Part II: Synthesis

As discussed in Chapter 2, dormancy is a widespread adaptation, found in all domains of life. Although the form and function of dormancy is not uniform, the ability to reduce metabolic activity, whether induced via internal signals, as in diapause, or in reaction to external stimuli, as in quiescence, is likely an early adaptation, and may have originated in the early, single celled ancestors of all living things as an adaptation to the resource limitations resulting from rapid generation times (O'Farrell, 2011). This early evolutionary origin makes its ubiquity understandable, and emphasizes its role as a basic adaptation in living things. Given these origins, it is not surprising that it would be found in a group as varied as the Foraminifera, which are evolutionarily closer to those ancestors than more complex, multicellular organisms such as metazoans. Nor is it a surprise, as suggested by the existing literature, that it seems to be a basic adaptation, widespread through the phylum. As some of the first non-propagule dormancy-focused research, the results of this dissertation emphasize the role of dormancy in *Amphistegina*

gibbosa and represent early steps in understanding both the mechanics and implications of dormancy in the Foraminifera.

Chapter 2 demonstrated, through the use of the CellTracker Green (CTG) fluorescent probe, that *A. gibbosa* exposed to propylene glycol, and lacking external signs of metabolism or activity, such as extrusion of pseudopodia or presence of waste, were nonetheless alive, showing signs of reduced metabolic activity, as indicated by the presence of CTG fluorescence. These methods do come with limitations. Propylene glycol interferes with CTG fluorescence, making direct incubation in propylene glycol with CTG ineffective, and requiring alternate methods that may limit its efficacy in identifying the effects of propylene glycol during exposure, instead only allowing observations of effects immediately after removal from exposure. In addition, symbiont autofluorescence masks the CTG signal, likely making determinations about metabolic activity less precise than they would be in the absence of symbionts. Still, these findings are consistent with the findings of Ross & Hallock (2014), that a recovery period was necessary following chemical bioassay treatments to identify individuals that were truly dead post-treatment.

The CTG experiments also indicated that *A. gibbosa* were able to survive up to 62 days in aphotic conditions. The survival of *Amphistegina* spp. in darkness had been observed previously. Lee et al. (1991) observed survival of *A. lobifera* following 13 weeks in darkness, and Smith & Hallock (1992) observed significantly longer survival of *A. gibbosa*, seeing recovery following return to light after up to 12 months in darkness, with the endosymbiotic diatom population surviving up to 18 months. Although I used the same methods in these darkness experiments as in the propylene glycol experiments for the sake of consistency, future experiments looking at dormancy in *A. gibbosa* may benefit from focusing on darkness over chemical exposure as an initiating factor, to avoid complications arising from chemical-CTG interactions.

The extreme differences in survival ability observed by Lee (1991) and Smith and Hallock (1992) pointed towards the presence of a reduced metabolic dormant state to facilitate long term survival. In light of observations made in this dissertation, the fact that the individuals in Lee et al. (1991) were removed from dark conditions for observation weekly may have limited the effectiveness of the dormant state. Increased CTG brightness in treatments removed from darkness and incubated in CTG in the light versus those incubated in the dark (Chapter 3) indicated that recovery can begin on time scales as short as 30 minutes. On the other hand, observations of symbiont-color recovery in Chapter 3 suggested that full recovery can take up to 99 hours, and observations of activity recovery in Chapters 4 and 5 showed that the time needed for recovery differed with the length of time the foraminifers were in darkness. These observations highlight the complexity of the mechanism, and it is possible that, in the experiments of Lee et al. (1991), the foraminifers were not able to reach or maintain a metabolically reduced state due to the experimental methods. This would explain why the foraminifers in the experiments of Smith & Hallock (1992), as well as those in the experiments described in Chapters 3 and 4 of this dissertation, which were not regularly removed for observation, were able to survive so much longer.

Another explanation could be that *A. lobifera* lacks the ability to go dormant in aphotic conditions, or possesses this ability in a reduced form compared to *A. gibbosa*. Given the apparent ubiquity of dormancy as a survival mechanism across the Phylum Foraminifera, as well as its presence as an early adaptation among single-celled organisms (as summarized in Chapter 2), the complete lack of the dormancy mechanism seems unlikely. This is especially true in light of its now well-documented presence in the closely related *A. gibbosa*. If dormancy is less effective in *A. lobifera*, a better understanding of the mechanisms involved with dormancy could

explain the difference. Moreover, understanding the differences in dormancy between two species of the same genus could help elucidate an ecological role of dormancy in the Foraminifera.

In this vein, the difference in extent of survival between that seen by Smith & Hallock (1992) and in the experiments presented in Chapters 3 and 4 of this dissertation highlights the role that the environment plays in a successful exit from dormancy and subsequent recovery. Smith & Hallock (1992) observed substantial recovery of the foraminiferal holobiont following 12 months in darkness, but only recovery of the color of the diatom endosymbionts after 18 months. In this dissertation, however, I observed 100% recovery of the holobiont following up to 12 months in darkness, with >80% recovery following 20 months in darkness. The likely explanation is my slow reintroduction of the foraminifers to lighted conditions. The light sensitivity of *A. gibbosa* is well documented (Hallock et al., 1986; Talge & Hallock, 2003; Williams & Hallock, 2004), and was seen in both Chapter 2, where the strong light associated with fluorescence microscopy caused damage to the foraminifers, and in Chapters 4 and 5, where damage associated with photic stress was observed and increased in extent the longer the foraminifers were held in darkness. Given these observations, it is likely that the extent of recovery in the field would depend heavily on the environment into which the foraminifers were reintroduced.

The observations of color recovery also reflect the health and management of the diatom endosymbiont population in a way that observations of foraminiferal activity recovery cannot. The presence of the endosymbiont population, and the apparent co-occurrence of dormancy in host and symbiont, as indicated by the recovery of symbiont-based color following removal from darkness, is something that the more recent studies of dormancy in the non-symbiont-bearing

Ammonia spp. could not address. Observations of symbiont autofluorescence made in Chapter 3 indicated a concentration of symbionts deeper in the cell. This interpretation was reinforced by observations presented in Chapter 3 that color recovery was faster and more complete in smaller individuals. The difference in color hue (Color Rank in Chapter 4 and 5) upon initial return to lighted conditions indicated a difference in photosensitivity depending on the length of time in darkness. When removed from the shorter 7- and 12-month treatments, foraminifers were lighter than their normal, healthy color, similar to those kept in darkness for 62 days in Chapter 3. This was consistent with a symbiont population concentrated away from the shell periphery. In the longer 15- and 20-month treatment populations, darker than normal coloration was observed more commonly, which correlates with photic stress. This is consistent with the observation that the longer treatments also exhibit lower percentages of the surface area colored (PSA of Chapters 4 and 5) and a higher incidence of mottling (as expressed by the Color Coverage Ranking, or CCR, of Chapters 4 and 5). Although dormancy did appear to co-occur between the foraminiferal host and its symbionts, extent of recovery was not necessarily the same.

The presence of mottling, even after a significant recovery period, could also indicate digestion of the symbionts or autolysis of cytoplasm by the foraminifers during dormancy. The CTG observations in Chapter 2 indicated the presence of metabolic activity, even if it was much reduced. Some level of autolysis may be necessary to drive this activity after lipid storage products are consumed; this would be consistent with the cytosol thinning observed by Koho et al. (2018) in *Ammonia.* The mottling, then, could have indicated areas of the cell that had been digested or even entire chambers of the shell in which the cytoplasm was digested and the chambers cordoned off analogous to observations by Talge & Hallock (2003). Thus, although photic stress was the most likely culprit of most of the observed differences in coloration, it may

also reflect some of the metabolic necessities of foraminifers that are neither actively feeding nor photosynthesizing. This metabolic requirement could be why the extent of recovery decreases as the time in darkness increases; some individuals may just lack the necessary intracellular resources to survive, even with highly reduced metabolic demands. This also suggests that larger, healthier individuals may be more capable of surviving extended periods of darkness. Comparing survival between healthy individuals and those grown under light limitations, which tend to be smaller and show lower thickness-to-diameter ratios (e.g., Hallock et al., 1986) could explore this hypothesis.

Taken as a whole, the observations from Chapters 2–5 form a picture of an *Amphistegina gibbosa* holobiont capable of incredible resiliency in the face of stressful environmental conditions and especially aphotic conditions, despite being obligate photosynthesizers. Some level of metabolic activity must be present while dormant, but is significantly lessened, allowing for survival for extended periods of time as long as conditions remain consistent. The onset of dormancy may be relatively rapid (the only documented time of onset in this dissertation being within 48 hours of exposure to propylene glycol) and the increase in metabolic activity associated with the return of normal conditions may be occurring on the timescale of minutes or hours, although active movement and feeding by the foraminifers may take a number of days to resume, depending on how long they were dormant. In the case of aphotic dormancy, then, this puts survival on the same time scale as that confirmed by experiments utilizing cryptobiotic propagules (as discussed in Chapter 1), and implies that post-propagule dormancy could be nearly as important in structuring foraminiferal assemblages and understanding foraminiferal ecology in stressful or marginal conditions.

Part III: Limitations and Further Research

A full understanding of dormancy in the *Amphistegina gibbosa*-diatom endosymbiont holobiont will require significantly more research. There are a number of gaps in current understanding of the basics of dormancy. For example, although evidence from CTG fluorescence microscopy suggested that onset of recovery from darkness can occur within half an hour, there were no direct observations concerning the time scale on which the foraminifers enter dormancy. It is possible that entry and exit may be occurring on a time scale small enough to account for daily variations, possibly even nightly. Without understanding the speed of onset, however, a full understanding of how the timing of dormancy entry and exit allows survival under stressful conditions is impossible. Similarly, how this timing differs between different initiating triggers, which could be useful as recognition of dormancy as a widespread survival mechanism increases, is unknown.

As discussed in Chapter 2, the use of CTG for identification of metabolic activity poses a number of difficulties when used with symbiont-bearing foraminifers. Other techniques may prove more beneficial in understanding the metabolic changes associated with entrance, maintenance, and exit from dormancy. Although destructive to the cell, ATP bioluminescence analysis is an obvious option. Although it would not allow for repeated sampling of the same individuals, it would allow for direct observation of average ATP presence in different replicates, and may give a more quantitative measure of metabolic activity than CTG.

Measurements of oxygen consumption would also be beneficial in understanding dormancy, especially in light of the work by Koho et al. (2018), who suggest that dormancy can be a strategy by which foraminifers minimize oxygen demand for survival in intermittently

anoxic sediment. Manipulation of light levels may even allow for the untangling of symbiont and host respiration. Work by Walker et al. (2011) and Mendez-Ferrer et al. (2018) have established methods for utilizing Clark-type oxygen electrode respiration systems with *A. gibbosa*, but these methods come with their own caveats. Due to the size of *A. gibbosa*, these methods call for at least 5 individuals in the respiration chamber to reliably pick up a signal. This makes it difficult to work with foraminifers under dormant conditions, because of the difficulty in distinguishing live from dead individuals. If one of the 5 assumedly dormant foraminifers is actually dead, the signal would be incomparable to other replicates, so methods would need to take this into account. In addition, the size of the electrode needed to pick up the signal is so large that the signal is relatively noisy, and the electrode itself consumes a significant portion of oxygen, making it impossible to observe changed in oxygen consumption over extended periods of time, as the seawater eventually becomes anoxic (i.e., over the 72-hour recovery period used in the propylene glycol exposure experiment in Chapter 2). With more sensitive electrodes, they could be a useful tool for observing changes in oxygen consumption as the foraminifers enter and exit dormancy.

How the cellular ultrastructure changes over time under dormancy, or how the relationship between host and endosymbiont changes is still unknown. Light and fluorescent microscopy suggests that the symbionts are being moved internally. The lag between color recovery in small and large individuals could also imply a level of symbiont reproduction necessary for full recovery post-removal from dormancy-initiating conditions. My observations of color recovery also suggest that isolated digestion of cytoplasm may be occurring, while observations of CTG fluorescence in propylene glycol-exposure treatments suggests the sequestration of CTG, and assumedly the propylene glycol media, in the outer chambers. This

may indicate the presence of apertural plugs, which might be recognizable as electron dense bodies under transmission electron microscopy. In fact, as shown by Nomaki et al. (2016), LeKieffre et al. (2017) and Koho et al. (2018), electron microscopy is ideal for observations of ultrastructural changes associated with dormancy. Unfortunately, despite a significant amount of effort and the use of previously established methods, I was unsuccessful in preparing *A. gibbosa* for TEM (see Appendix III for summary of methods). Although *A. gibbosa* poses a number of difficulties in TEM preparation, including the size of the cell and thickness/porosity of the shell, further refinement of methodology and technique could allow consistent preparation of *A. gibbosa* for TEM analysis. This, in combination with techniques such as correlative NanoSIMS, as discussed above, could answer many of these questions concerning ultrastructural changes, and could be especially helpful in understanding the interactions between host and symbiont on a very fine spatial resolution under different conditions, dormancy included.

The use of NanoSIMS (e.g., Nomaki, 2016; LeKieffre, 2017) may offer a way forward for the study of dormancy in the Foraminifera in general. As discussed above, additional, relatively low-resolution work could aid- understanding entry and exit times, identifying triggers, or something as simple as identifying dormancy in reaction to known triggers in more taxa. These would all be useful from an ecological perspective, especially in terms of assemblage interpretation. Such experiments could be more useful than the finer scale cellular methods for some applications, for instance, paleoceanographic reconstructions using foraminiferal assemblages.

However, to understand what drives dormancy and how it functions, as well as whether it is functionally different depending on initiating trigger, a more detailed understanding of the cellular processes is needed. The NanoSIMS applications discussed above are a good example of

how different signals, in those cases, molecular and elemental sequestration, can be interpreted to draw conclusions concerning differences in activity at very fine scales when the foraminifers are exposed to stress.

Another possible technique with wide ranging implications for understanding stress in Foraminifera in general, and dormancy in particular, is by studying how the proteome changes. Proteomics may offer clues to the stress-related proteins that provide the signal to trigger the onset of dormancy. They may also allow for the identification of proteins that functionally drive the entry, maintenance, and exit from dormancy. Once the protein sequences and functions are identified, they could allow for identification of taxa capable of dormancy via gene sequencing, making exploring the extent of dormancy in the Foraminifera easier. It could also allow for the identification of those proteins in environmental samples, or in specimens in laboratory experiments, making it easier to determine whether foraminifers are living or dead.

Evidence points towards dormancy being considerably more common in the Foraminifera than was recognized until recently. As it becomes better understood and more widely recognized, dormancy has the capability to substantially enhance understanding of foraminiferal ecology, with wide ranging implications for the many applications utilizing foraminifers in some capacity. Further research concerning dormancy at multiple resolutions, using a variety of techniques, will continue to change understanding of how foraminifers react to stress, and can only improve on researchers' ability to interpret the patterns seen in the geologic past, in the present, in the laboratory, and in predictions for the future.

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APPENDIX I:

DORMANCY IN THE FORAMINIFERA: A REVIEW

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DORMANCY IN THE FORAMINIFERA: A REVIEW

BENJAMIN J. ROSS* AND PAMELA HALLOCK

College of Marine Science, University of South Florida, 830 1st Street S., St. Petersburg, FL 33701 USA

ABSTRACT

The Foraminifera are a diverse class of protists whose ubiquity in marine environments, small shells, and ease of collection have made them critical tools in bioindicator, bioassay, paleoenvironmental, and paleoceanographic research. Despite the plethora of applications and accompanying literature on foraminifers, many aspects of their biology and ecology remain unexplored. One of these aspects is dormancy, a life-history strategy involving suspension of active life, arrested development, and reduced or suspended metabolic activity, mediated either by internal physiological factors (known as diapause) or exogenous factors (known as quiescence). Dormancy is a widespread adaptation, playing a role in the life cycles of a huge variety of organisms. Yet, despite anecdotal and circumstantial evidence, very little research has directly addressed this aspect of foraminiferal biology. The relatively recent discovery of cryptobiotic propagules has revealed a fundamental role for dormancy in the life cycles of foraminifers, most prominently for dispersal. Moreover, culture studies with environmental applications have shown that post-propagule quiescence (i.e., in juveniles, sub-adults and adults) may be a common response to environmental stressors, allowing rapid recovery of populations following disturbance or otherwise unfavorable conditions.

A review of publications on foraminiferal biology revealed that observations indicating the potential for dormancy have been recorded for at least six decades, and that this potential is well represented throughout the class in a variety of forms, suggesting that dormancy may be a basic adaptation in the Foraminifera. If dormancy is as widespread as the literature suggests, its role in structuring foraminiferal assemblages and determining global distributions in the geological past, present, and future is fundamental. Further research into the mechanisms of dormancy will expand understanding of its role in foraminiferal life cycles, and provide new perspectives in the many fields that utilize and apply foraminiferal data.

INTRODUCTION

Members of the protistan Phylum Foraminifera (d'Orbigny, 1826) (phylum status was proposed by Mikhalevich, 2004 and Pawlowski et al., 2013) are ubiquitous in marine environments. They are well preserved in Phanerozoic sediments and sedimentary rocks as a consequence of their organic, agglutinated, or calcareous shells (commonly called "tests"), making them among the most widely utilized organisms in earth and ocean sciences (e.g., Sen Gupta, 1999). Their shells are the second most abundant component (after coccoliths) of the calcareous marine sediments that cover roughly half the ocean floor (Kennett, 1982). The Foraminifera are essential to facets of environmental, evolutionary, sedimentological, paleoenvironmental, micropaleontological, biostratigraphic and paleoceanographic research, and continue to be widely used in economic applications. Historically, research has focused on the preserved shells rather than the live organisms (e.g., Sen Gupta, 1999). Goldstein (1999) estimated that, of >10,000 extant species, relatively complete life cycles are known for <30.

One aspect of foraminiferal biology that remains poorly understood is the occurrence and prevalence of dormancy as a life strategy. In her biological overview, Goldstein (1999) did not mention dormancy. Despite a wealth of anecdotal and circumstantial evidence, very little research has directly addressed this aspect of foraminiferal biology, the understanding of which has implications in all fields of research utilizing the Foraminifera, and well beyond. The goals of this paper are: 1) to provide a brief introduction to the topic and terminology of dormancy; 2) to review the published literature in which dormancy in foraminifers has been observed directly, or in which the presence of a dormant state is inferred by the authors based on the observed evidence; 3) to present evidence that dormancy has been observed across a wide range of benthic taxa, in contrast to limited evidence but broad implications for planktic taxa; and 4) to propose ways that recognition of dormancy as a basic biologic mechanism can be used to interpret the past and to design future research.

TERMS AND DEFINITIONS

The term "dormancy" describes a life-history strategy that encompasses a wide variety of physiological states, which can differ significantly regarding initiation triggers and how it is expressed (Cáceres, 1997). What these states tend to have in common is that they involve a suspension of active life, arrested development, and reduced or suspended metabolic activity (i.e., Guidetti et al., 2011; Lennon & Jones, 2011). Even then, Clegg (2001) points out that researchers have historically had difficulty agreeing on whether metabolic activity can be truly suspended or just highly reduced. Dormancy strategies can be broadly divided into two categories: quiescence, which is induced by exogenous factors (i.e., a reaction to stressful environmental stimuli); and diapause, which is induced by internal physiological factors, and which may or may not be related to environmental conditions (Cáceres, 1997; Guidetti et al., 2011).

Evidence for quiescence is considerably more common in the foraminiferal literature than for diapause (Table 1). Research bias likely plays a role in this difference. Because few studies have directly explored dormancy, let alone diapause specifically, dormant stages have tended to be encountered in reaction to environmental alterations, intended or otherwise. Similarly, if a diapausing organism enters dormancy in reaction to regular disturbance (i.e., seasonal changes), dormant stages encountered during instantaneous sampling would likely be attributed to the disturbance, regardless of whether the disturbance itself triggered dormancy. Despite this, there is some evidence for diapause in the Foraminifera, making the distinction important.

*Correspondence author. E-mail: benjaminross@mail.usf.edu

TABLE 1. Summary of previously reported evidence for dormancy among the Foraminifera.

Type of dormancy	Taxon/taxa observed	References
Diapause		
Seasonal dormancy	Rotaliida (*Elphidium crispum*)	Myers (1942, 1943)
Seasonal resistant cyst formation	*Reticulomyxa filosa*	Gothe et al. (1997)
Quiescence		
Response to reduced temperature (Cryobiosis)	Rotaliida (*Ammonia tepida*)	Bradshaw (1957)
Response to anoxia (Anoxybiosis)	Buliminida, Lituolida	Bernhard (1993), Bernhard & Alve (1996), Moodley et al. (1997)
	Rotaliida, Miliolida, Textulariida	Moodley et al. (1997)
	Astrorhizida (*Astramina rara*)	Bernhard (1993)
Response to anoxia and reducing conditions (presence of H_2S)	Rotaliida, Buliminida, Lituolida, Astrorhizida, Miliolida	Bernhard (1993)
	Buliminida, Lituolida, Astrorhizida, Miliolida, Textulariida	Langlet et al. (2013, 2014)
Response to chemical exposure	Rotaliida (*Amphistegina gibbosa*)	Ross & Hallock (2014), McCloskey (2009)
Response to extended darkness	Rotaliida, Miliolida	Smith & Hallock (1992)
Cryptobiosis/resistant propagules	Rotaliida	Alve & Goldstein (2002, 2003, 2010), Goldstein & Alve (2011)
	Astrorhizida, Allogromiida, Trochamminida, Miliolida, Lagenida	Goldstein & Alve (2011)
	Lituolida, Textulariida, Buliminida	Alve & Goldstein (2010), Goldstein & Alve (2011)
	Globigerinida	Nigam (2005)
Resistant cysts		
In response to anoxia	Rotaliida	Linke & Lutze (1993)
In response to anoxia and reducing conditions	Buliminida	Bernhard (1993)
Function uncertain	Rotaliida, Lituolida, Trochamminida	Heinz et al. (2005)
	Unidentified unilocular and agglutinated species	Heinz et al. (2005)
Uncovered resting stages	*Reticulomyxa filosa*	Gothe et al. (1997)

Under these two broad categories, numerous dormancy strategies are recognized. Extreme quiescence is known as cryptobiosis, or "hidden life", defined as a state in which an organism "shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill" (Keilin, 1959, p. 166). This state is further involved in a number of forms of dormancy. Keilin (1959) notes that the earliest recorded discussion of cryptobiosis was related to anhydrobiosis (desiccation), first identified by Antony van Leeuwenhoek in 1702, but also notes that cryptobiosis can result from other environmental pressures such as low temperature (cryobiosis) or lack of oxygen (anoxybiosis). In a review focusing only on invertebrates, Cáceres (1997) also identified osmobiosis (water potential), hibernation, and estivation as triggers for dormancy, describing a variety of specialized resting stages, resting eggs, and dormant cysts. In just the Phylum Tardigrada, numerous dormancy strategies are known. Diapause is present in the form of encystment, resting eggs and cyclomorphosis, while quiescent cryptobiosis is represented by anhydrobiosis, cryobiosis and anoxybiosis (Guidetti et al., 2011).

Dormancy of some form is widely represented in living organisms. O'Farrell (2011) considers cellular quiescence a primitive biological process and suggests that its early evolution was necessary to survive resource limitations inherent to rapid generation times. In metazoans, cellular quiescence has been further adapted and plays a key role in size control both of organisms as a whole and of specific tissues; disruption of quiescence is a key process underlying cancer.

Dormancy is widespread as a life-history strategy at the organismal level. Among plants, plant seeds exhibit anhydrobiosis, as does the vegetative tissue of "resurrection plants", which include representatives across several plant divisions (i.e., Bewley, 1997; Clegg, 2001; references within both). In addition to dormancy in seeds, dormancy can be found in individual organs, such as buds on higher plants (Vegis, 1964); Rohde & Belerao (2007) place the regrowth of perennial plants in the context of dormancy. Dormancy is also present in a variety of vertebrate phyla, from hibernating mammals such as polar bears (i.e., Lennox & Goodship, 2008), hedgehogs, bats and rodents (Barnes, 1970; Storey, 2010); reptiles such as painted turtles (Storey et al., 1988; Jackson, 2002) and possibly sea turtles (Felger et al., 1976); amphibians (e.g., Seymour, 1973); and both larval (Podrabsky et al., 2010) and adult fish (Crawshaw, 1984), including lungfish (Smith, 1930). Cáceres (1997) reviewed research on dormancy strategies in invertebrates, finding evidence for its presence in 16 phyla.

Dormancy is certainly widespread, if not ubiquitous, within the Kingdom Protista, which includes a large and diverse array of eukaryotic microorganisms (e.g., Corliss & Esser, 1974). Arguably the most important protists on Earth are the photosynthetic groups whose representatives include the phytoplankton and some macroalgae. Cyst formation is recognized among, for example, both dinoflagellates (e.g., Binder & Anderson, 1990; Figueroa et al., 2007; Smayda & Trainer, 2010; Lundholm et al., 2011; Bravo & Figueroa, 2014) and diatoms (e.g., Smetacek, 1985; McQuoid & Hobson, 1996; Lewis et al., 1999; von Dassow & Montresor, 2010). Other protistan groups in which dormancy mechanisms are recognized include some of the most economically costly human parasites (e.g., *Plasmodium* spp. that cause malaria) and others that cause widespread damage to

agricultural industries. Spore formation and other dormancy mechanisms are a basic strategy in the life histories of parasitic sporozoans (e.g., Melhorn & Heydorn, 1978).

Given the broad range of forms that dormancy can take, and the wide variety of biological settings in which it is found, it is not surprising to find evidence of dormancy in the Foraminifera. What is more surprising is the relative dearth of documentation thereof.

EVIDENCE FOR DORMANCY IN THE FORAMINIFERA

Early Studies

Early mention of dormancy in a foraminiferal taxon can be found in two papers by Myers (1942, 1943) discussing the life cycle of the benthic foraminifer *Elphidium crispum* (Linné). Sampling monthly from tide pools and the sublittoral zone around Plymouth, England, Myers (1942) found significant protoplasmic shrinkage in specimens collected in mid-winter, followed by recovery and refilling of the shells through February and March. No shrinkage was observed in specimens collected during the summer or fall. Myers interpreted this shrinkage as the result of seasonal dormancy (Table 1) in response to limited food sources, but not to temperature, since the period of greatest growth and reproduction occurred following the coldest period of the year.

When food became available, Myers (1942) noted that foraminifers recovered; the timing of recovery depended upon differences in the bottom turbulence required to unbury *E. crispum* dormant in the sediment. Myers considered dormancy to be a key factor of the *E. crispum* life cycle, with resource-mediated winter dormancy followed by a period of growth and sexual reproduction, eventually followed by asexual reproduction.

Sampling at other sites around Plymouth supported this interpretation (Myers, 1943). At the Wembry site near the entrance to Plymouth Sound, hydrographic conditions in early spring scoured the substratum and delayed regrowth of phytoplankton that serve as *E. crispum*'s food source. Compared to other sites at the same time of year, the frequency-distribution curves for growth stages in *E. crispum* for samples taken at Wembry indicated a reduction in growth and an extension of the reproductive period, interpreted as the result of a delayed exit from dormancy. Myers thus identified the dormant stage as what would now be recognized as starvation-linked quiescence, as opposed to a seasonal diapause. Since the specimens observed were killed following collection, this conclusion remains a matter of interpretation of the data. Myers (1943) discussed the possibility of testing for and observing cryobiosis in *E. crispum* once successful culture techniques were developed, but ultimately considered the possible results as being of "little significance", since the coldest temperatures observed at his study sites did not correlate to the period of winter dormancy. Despite the lack of direct evidence, Myers' observations have been discussed in a number of contexts, including their importance to the interpretation of paleoceanographic data from sediment cores, where the yearly cycle of dormancy was suggested as contributing to a recurrent cycle of calcareous foraminifers observed in laminated Miocene well cores (Riveroll & Jones, 1954), and as playing a role in understanding foraminiferal population dynamics (Erskian & Lipps, 1987).

Bradshaw (1957) exposed cultures of 6–10 specimens of *Ammonia tepida* (Cushman) [identified as *Streblus beccarii* (Linné)] to temperatures ranging from 10–30°C to observe the effects of temperature on reproductive behavior and the rate of chamber addition. What he reported is a possible example of cryobiosis, although not identified as such. In samples kept at 10°C, no observable growth was recorded, but the specimens did not appear to die, and instead continued to live without reproducing. Following 27 days at reduced temperatures, the specimens were transferred to media kept at room temperature of 24–27°C, commencing rapid growth, and reproduction within 25 days. Bradshaw did not address measures of metabolism and vitality in these specimens. As such, it cannot be determined whether this is an example of cryobiosis, a dormant stage entered as a survival strategy, or a slowing of metabolic activity caused by living well below optimum temperatures. Experimental treatments of increasing temperature produced a gradient of increasing activity (Bradshaw, 1957). Samples kept at 15°C grew slowly, and did not reproduce until moved to warmer temperatures. Samples kept at 20°C had reproductive difficulty, with abortive attempts at reproduction and failure of normal shell development in the offspring, and no successful reproduction until at least 76 days after the start of the experiment, although growth rates were not mentioned. Meanwhile, samples kept at room temperature of 24–27°C showed growth to reproductive maturity in 19 days; at 30°C reproduction occurred within 15 days. Whether or not Bradshaw (1957) observed *A. tepida* truly entering a cryobiotic dormant state, these results suggest that decreased metabolism in cold temperatures and subsequent recovery and resumption of regular life activity is possible in these foraminifers. This stands in contrast to Myers (1942, 1943), who did not see any evidence of temperature-mediated dormancy in *E. crispum*.

More Recent Studies

As in the early studies, the majority of recent evidence for dormancy in the Foraminifera is concerned with benthic species. Only one paper was found that addressed the topic of dormancy in planktic foraminifera. These recent reports fall into one of two categories. The first concerns evidence of cryptobiotic propagules, which are tiny individuals produced either asexually or sexually that are resistant to unfavorable conditions and that aid in dispersal. The second category is evidence of dormancy strategies as a survival mechanism for benthic foraminifers in post-propagule life stages.

Cryptobiotic Propagules

Evidence for cryptobiosis was first documented by Alve & Goldstein (2002, 2003), and further explored in subsequent papers (Table 1). Samples of sediments, which had been collected at depths >140 m and that passed through 32 μm mesh sieve, were maintained in sealed containers in a darkened cold room for up to 4 months. When those sediments were placed in simulated shallow-water conditions for at least 3.5 weeks, live *Haynesina germanica* (Ehrenberg) and

Ammonia beccarii (Linné) with diameters >63 μm were observed (Alve & Goldstein, 2003). These species typically occur in shallow water and no live individuals were found in replicates examined immediately after collection. The presence of nearly spherical, agglutinated, cyst-like structures, some of which contained healthy-appearing *H. germania*, as well as the fact that all individuals emerged from the <32 μm size fraction of the original sediment, indicates that they had been present in the original sediment as small, possibly encysted propagules. The ability of these propagules to survive in environments where adults are not found, and to resume growth under favorable conditions, may be a fundamental mechanism of dispersal. Moreover, the presence of both microspheric and megalospheric juveniles indicates that propagules can be produced either sexually or asexually. These observations were recognized as evidence for cryptobiosis by the authors (Alve & Goldstein, 2003).

Subsequent experiments (Alve & Goldstein, 2010) revealed a diverse assemblage of shallow-water benthic foraminifers apparently surviving as propagules in sediment taken from depths >320 m in the North Sea, and capable of growth once the sediment was placed in simulated shallow-water conditions. The assemblage observed to grow from silt-sized sediment (<32 μm) placed in shallow-water conditions soon after collection was the same as that observed in sediment kept in sealed containers in a dark coldroom for two years before being placed in simulated shallow-water conditions. Thus, the cryptobiotic propagules of these species appear to be capable of surviving at least two years in inhospitable conditions (Alve & Goldstein, 2010). Adaptation of these methods and exposure of the fine-grained (<53 μm) sediment fraction from mudflats on Sapelo Island, Georgia, to experimental treatments of differing salinity and temperature resulted in the growth of different assemblages depending on environmental conditions (Goldstein & Alve, 2011). These results demonstrate that a diverse, dormant-propagule bank allows foraminiferal assemblages to react rapidly to changing environmental conditions.

Observations in post-propagule life stages

Encystment. Although the previously cited papers were concerned with encystment of propagules, encystment in foraminifers has been known for decades and seems to serve many purposes. For example, Jepps (1942) discussed the role of cysts in feeding (and possibly protection) in *Elphidium* (identified as *Polystomella*) Lamarck. Angell (1990) described the formation of protective reproductive and early growth cysts in *Trochammina inflata* (Montagu). Linke & Lutze (1993) observed that *Elphidium incertum* (Williamson), which lives infaunally in the upper sediment layers, could be found in agglutinated cysts at depths of 3–6 cm, as well as in anoxic sediment, with no sign of shell dissolution, suggesting that encystment is a survival mechanism in stressful and corrosive environments.

In laboratory observations of migrational activity of bathyal benthic foraminifera, Gross (2000) observed that some species, including *Gavelinopsis translucens* (Phleger & Parker), *Planorbulina mediterranensis* d'Orbigny, and *Rosalina floridana* (Cushman) demonstrated intermittent resting stages or built dormant cysts, in which they would rest for periods of weeks or months before the cysts were abandoned and new ones formed. As these observations were made in non-experimentally manipulated chambers, there is no suggestion that the cysts were formed in reaction to environmental conditions, and Gross does not identify them as feeding or reproductive cysts, suggesting these dormant cysts could be an indication of diapause-related dormancy. Gross (2000) also suggests that dormant stages or cysts allowed some species to tolerate experimentally induced dysoxic conditions, in which actively moving foraminifers were rarely observed. Gross did not name species observed in dormant stages or that encysted in this experiment.

Encystment in adult benthic foraminifers was reviewed and explored by Heinz et al. (2005). They summarized evidence for the formation of a variety of cysts, including feeding, reproductive, growth, and protective cysts, in the literature. Heinz et al. also observed, in laboratory conditions, the formation of cysts in numerous taxa, including organic-walled, agglutinated, and calcareous species. Dormancy was not mentioned in the reviewed papers, and with the exception of one case in which reproduction took place, Heinz et al. were unable to discern the function of cysts that they observed. In addition, there was no discussion of metabolic depression of encysted foraminifers. However, these results reinforce the prevalence of encystment among foraminifers of different taxa and age classes, indicating that encystment-associated dormancy is possible in many species.

Gothe et al. (1997) discussed the presence of resting stages and thick mucosal organic cysts in *Reticulomyxa filosa* Nauss. This freshwater protist lacks a shell, but has long been considered a relative of the Foraminifera. Based on genetic studies, Pawlowski et al. (1999a, b) concluded that it should be recognized as a member of the Foraminifera. Gothe et al. (1997) reported that *R. filosa* developed uncovered resting stages when environmental conditions deteriorated (i.e., low temperatures or deterioration of unreplenished culture media following overgrowth of other organisms). Although not resistant to environmental extremes such as sub-zero temperatures, desiccation, or air contact, the specimens survived for two to three weeks in the unfavorable environment, and recovered within minutes upon the improvement of conditions. Moreover, all specimens in a culture developed uncovered resting cysts in synchrony when conditions deteriorated. Many *R. filosa* specimens formed covered cysts (with thick bilayered walls) in summer cultures, regardless of media, suggesting seasonality that may be linked to an increased likelihood of desiccation in summer temperatures. Although not all individuals formed these cysts, this observation indicates an endogenous trigger for cyst formation, which is an example of diapause. The dried cysts were able to survive complete desiccation, freezing and application of pressure, and they survived up to 23 days and recovered within 2–3 days when media was added. Much like juvenile propagules, these cysts may be a key factor in the dispersal of *R. filosa*, suggesting diapause is an integral, possibly obligate, life stage. Cáceres (1997) noted that dormancy is more common in freshwater and terrestrial species in taxonomic classes that include marine species, and may be an adaptation to cope with variable conditions. However, dormancy in *R. filosa*

does not reveal information about the evolutionary origins of dormancy in the Foraminifera, because, as Pawlowski et al. (1999a, b) reported, *R. filosa* is not a shell-less basal foraminifer, but instead is a highly adapted taxon that has lost its shell.

Anoxia. Hypoxia and anoxia are major environmental stressors for which evidence indicates dormancy as a survival strategy in foraminifers. Linke & Lutze (1993) observed cysts associated with anoxic conditions. Hannah & Rogerson (1997) reported that live foraminifers buried in anoxic benthic sediment (e.g., via bioturbation) may survive in an "inactive" state from which they can recover in a relatively short amount of time after return or relocation to favorable conditions. The study's reliance on rose Bengal staining, which is not effective at differentiating live and dead cells on short time scales (Bernhard et al., 2006), makes it difficult to draw conclusions about the viability of buried foraminifers. However, the ability to become dormant in anoxic sediments subject to frequent bioturbation would be an effective survival strategy.

Bernhard (1993) subjected benthic foraminifers collected from Explorer's Cove, Antarctica, to 4-week long experimental anoxic treatments. Using an ATP assay to determine vitality, she found no significant difference in survivorship or average ATP content, indicating that the observed foraminiferal species were capable of surviving at least four weeks in anoxic conditions. Bernhard noted some ultrastructural evidence for encystment, and also suggested metabolic depression (dormancy) as a survival mechanism. Bernhard & Alve (1996), performing similar experiments with species collected from Drammensfjord, Norway, performed ATP extraction within the anoxic treatments. They found that, in some species, survival was not affected by anoxic conditions, but that ATP was significantly depleted. A possible interpretation is that these species were surviving anoxic conditions by becoming dormant, with the reduced ATP indicating a reduction in metabolism, as opposed to groups that showed a decrease in both survival and ATP (dying or damaged under anoxic conditions) or no change in either (apparently capable of anaerobic respiration). Ultrastructural examination revealed withdrawn pseudopodial networks, indicating that the foraminifers were not actively feeding, thereby supporting this interpretation. Citing these results, Moodley et al. (1997) suggest dormancy may have contributed to the observed survival of hard-shelled foraminifera (predominantly *Nonionella*, *Stainforthia*, *Bolivina*, *Hopkinsina*, and *Reophax*) under experimentally-induced anoxic conditions.

More recently, Langlet et al. (2013) performed an in-situ experiment to observe the responses of epifaunal and infaunal foraminiferal assemblages at a shallow-water site in the Adriatic Sea. Using Experimental Anoxia Generating Units (EAGU), 0.125m^3 chambers placed on the seafloor, sediment was isolated from the surrounding water. Microelectrodes in the chambers indicated that hypoxia was reached after 2–6 days, anoxia after seven, and that the chambers remained anoxic for the duration of the experiment, up to 10 months. Foraminiferal assemblages from different depths in cores taken from within the chambers were incubated in CellTracker Green CMFDA (CTG). This nonfluorescent probe forms a fluorescent compound when hydrolyzed with nonspecific esterase, making identification of live organisms under an epifluorescent microscope possible (Bernhard et al., 2006). Although overall densities decreased, CTG analysis by Langlet et al. (2013) showed survival of foraminifers of all observed species following 10 months of in-situ anoxia. This included survival in species not known to store or respire nitrates, suggesting that anaerobic metabolism may not be responsible (as further discussed in Langlet et al., 2014). The authors suggested drastically decreased metabolic rates as a possible explanation for survival.

Other stimuli. Evidence for dormancy in foraminifers is most prominently found in studies of juvenile cryptobiosis and anoxia. Nevertheless, evidence for quiescence induced by other environmental stimuli can be found in the published literature.

Smith & Hallock (1992) presented observational results concerning the effect of prolonged darkness on foraminifers with algal symbionts. *Amphistegina gibbosa* d'Orbigny and *Archaias angulatus* (Fichtel and Moll) (subsequently genera are abbreviated *Am.* and *Ar.*, respectively) were placed in filtered seawater in opaque containers and subsampled at intervals ranging from a week to more than a year. Some *Am. gibbosa* (Order Rotaliida), which host diatom endosymbionts, survived darkness and regained normal symbiont color and behavior when returned to a light/dark cycle after as much as 12 months in the dark with no food source. *Archaias angulatus*, a chlorophyte-bearing member of the Order Miliolida, exhibited some recovery from prolonged darkness for up to three months.

Ross & Hallock (2014) documented recovery of *Am. gibbosa* following 48-hr exposures to propylene glycol and 2-butoxyethanol, which are ingredients in some hydrocarbon dispersants. Vitality of the foraminifers was assessed visually using a stereomicroscope; individuals that showed no attachment to the petri dish or no extended reticulopodia initially were considered dead. Immediately following treatment, the concentration that appeared to have killed 50% of the experimental population was 3% volume/volume (v/v) for propylene glycol and 0.2% v/v for 2-butoxyethanol. Following a 72-hr recovery period in filtered seawater, many individuals showed recovery of normal color and resumption of reticulopodial activity, such that the treatment concentrations that actually killed 50% of the experimental population were 6% v/v and 1% v/v, respectively.

By recording the mean daily growth of the surviving experimental populations for 40 days following removal from the propylene glycol treatment, Ross & Hallock (2014) observed a decrease in growth at low concentrations of propylene glycol where no inactivity was induced, when compared to medium concentrations in which inactivity was observed and in which subsequent recovery occurred. We hypothesized that this pattern was related to dormancy. If there is a threshold of chemical toxicity that triggers dormancy, which is not met at the low concentrations, those foraminifers may survive the immediate exposure to low toxicity but are negatively affected on a longer time scale. In contrast, the intermediate concentrations triggered dormancy, thereby decreasing the effects of the chemicals. The highest concentrations apparently killed too quickly for the onset of dormancy to occur.

McCloskey (2009) observed similar apparent mortality in experiments in which he exposed *Am. gibbosa* to arsenic (As). In an initial experiment, in which he was determining the range of As concentrations for subsequent experiments, specimens in 200 and 1000 µg/kg As^{3+} treatments and 1000 µg/kg As^{5+} treatments appeared to be dead after one week exposure. The specimens were then placed in clean seawater and the original treatment groups were monitored. After 4 weeks, specimens from the 1000 µg/kg As^{3+} treatments exhibited neither growth nor reticulopodial activity, while specimens in the 200 µg/kg As^{3+} treatments and the 1000 µg/kg As^{5+} treatments had regained normal color and had grown significantly. Given the results reported by Ross (2012) and Ross & Hallock (2014), McCloskey (2009) likely observed stress-induced dormancy in the 200 µg/kg As^{3+} and the 1000 µg/kg As^{5+} treatments.

Planktic Species

Although most evidence for dormancy in the Foraminifera has been found in benthic species, Nigam (2005) speculated that it may play a role in the life cycle of planktic taxa as well. Analyzing data from previously published sediment trap records from three sites in the Arabian Sea, as well as from sediment traps in other oceans, Nigam found seasonal fluxes of near zero or zero planktic foraminiferal shells. He also observed a seasonally rhythmic pattern of larger and smaller size fractions of the same species, with peaks of larger size fractions slightly preceding peaks of smaller size fractions. Nigam interpreted these trends as evidence for periods of dormancy alternating with the occurrence of reproduction during monsoon-associated favorable conditions.

Summary of Recent Evidence

A review of the evidence for the occurrence of dormancy in the Foraminifera (Table 1) indicates its presence in 10 of the 15 extant orders recognized by Sen Gupta (1999), as well as in *Reticulomyxa filosa,* now recognized as a member of the Foraminifera (Pawlowski, et al., 1999a, b). These orders represent a wide diversity of foraminiferal forms and habitats. They include mostly marine, benthic taxa, as well as a freshwater species, *R. filosa.* They also include organic-walled taxa, taxa with agglutinated shells utilizing both proteinaceous and mineralized cement, and taxa with both porcelaneous and hyaline calcite shells, as well as single-chambered taxa and large, symbiont-bearing taxa. The presence of dormancy in such a large variety of taxa, across the range of complexity in foraminiferal lifestyles, suggests that dormancy is a basic adaptation in the Foraminifera (Fig. 1).

IMPLICATIONS OF DORMANCY IN THE FORAMINIFERA

The study of dormancy in the Foraminifera has broad and fundamental implications for research and observations ranging from basic ecology and population dynamics, to understanding responses of assemblages to local, regional and global environmental changes. Foraminiferal distributions, for example, are far better understood in the context of tiny, cryptobiotic propagules (Alve & Goldstein, 2003, 2010) that can be widely distributed by waves and currents.

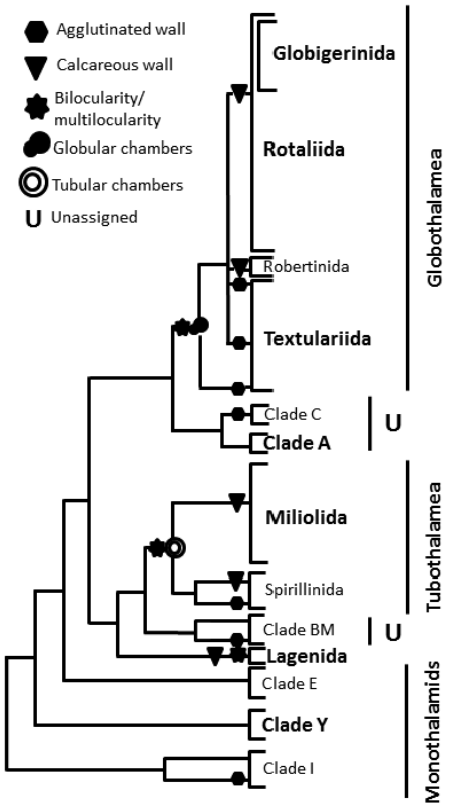

FIGURE 1. Ubiquity of dormancy in currently published supraordinal classification of Foraminifera based on SSU rDNA phylogeny. Names in bold indicate groups for which dormancy has been previously reported. Unassigned taxa have not yet been assigned to either of the classes Globothalamea or Tubulothalamea or to the paraphyletic monothalamid assemblage (modified from Pawlowski et al., 2013).

A testable hypothesis may be that cosmopolitan species produce propagules with great potential for dispersal, while endemic taxa may have lost this basic characteristic.

Recognition that cryptobiotic propagules are a fundamental aspect of foraminiferal biology allows researchers to interpret a vast array of ecologic and biogeographic observations, and to formulate hypotheses to test those interpretations. Myers's (1943) observation that food availability can be more important than temperature in stimulating emergence from dormant states has wide implications for understanding the ecology of foraminifers in the deep sea, where sporadic food availability such as whalefalls (e.g., Gooday & Rathburn, 1999; Lundstein et al., 2010) can trigger blooms.

Much like the deep sea, at high latitudes, though sunlight and water temperature may change in surface waters, the major environmental change is availability of organic matter sinking from surface-water phytoplankton blooms (e.g., Gooday et al., 1990; Gooday & Rathburn, 1999; Suhr et al., 2003; Suhr & Pond, 2006). DeLaca et al. (1980) observed that giant, arborescent, agglutinated foraminifers in the Antarctic became active in the presence of food. An interesting question, therefore, is: in an encysted or otherwise quiescent foraminifer, what sensory processes remain sufficiently active to detect the presence of food?

Duffield et al. (2014) explored the question of how propagules respond to different sources of food. They isolated the <53-μm size fraction from surface sediments collected from 355 m in the Outer Oslofjord, Norway, then added different food sources to subsamples of the sediment. Food sources included monospecific algal cultures and both phytoplankton and zooplankton detritus from net hauls. After six weeks, they sieved the sediment through a 63-μm mesh, allowing identification of any foraminifers originally present that had grown by at least 10 μm. Foraminiferal specimens >63 were recovered from all treatments, including those to which no food was added. Some food sources actually suppressed growth in most foraminiferal species present, while addition of monocultures of selected diatoms stimulated growth in some species. Experiments such as this can provide insight into the preferred food sources. Combined with assessment of taxa present in the original sediment using environmental genomics (e.g., Pawlowski et al., 2011, 2014a, b), there is much to learn about the food requirements of benthic foraminifers.

Propagules may also play a role in the life cycles of planktic foraminifers, as Nigam (2005) postulated. The awareness of the possibility of tiny cryptobiotic propagules as an important life stage in planktic foraminifers suggests a whole range of testable hypotheses. Might environmental-molecular-genetic surveys (e.g., Habura et al., 2004; Pawlowski et al., 2011) of pycnocline water samples reveal the presence of planktic foraminiferal propagules that accumulate at water-mass interfaces? Such accumulations could explain, for example, how *Globigerina bulloides* d'Orbigny and some other planktic taxa can quickly appear during upwelling or deep mixing events that trigger phytoplankton blooms (e.g., Rigual-Hernández et al., 2012, and references therein). Might gametogenetic calcification provide a mechanism to ensure concentrations of propagules at specific density gradients in pelagic environments? Moreover, what chemical stimuli in the environment and sensory mechanisms in the dormant propagules can trigger an increase in metabolic activity resulting in growth and reproduction? These are a few of the fundamental questions that emerge with the recognition of the possibility that planktic foraminifers can produce cryptobiotic propagules. Such questions can be addressed using a variety of experimental, physiological, and genomic approaches.

Food availability, however, is only one of the kinds of stimuli that can induce either inactivity or emergence from inactivity. Clearly, emergence from post-propagule quiescence has been recognized as a response to change in environmental conditions, such as food availability (Myers, 1942, 1943; DeLaca & Lipps, 1980), oxygenation (Hannah & Rogerson, 1997; Bernhard, 1993, Bernhard & Alve, 1996; Langlet et al., 2013), or reduction in exposure to a toxic chemical (Ross, 2012; Ross & Hallock, 2014). Given that hydrogen sulfide (H_2S) is a naturally occurring and very common environmental "toxin", a testable hypothesis is that the response to exposure to some other toxic chemicals (e.g., as observed by Ross & Hallock, 2014) may be analagous to the response to H_2S. Moreover, emergence from darkness-induced dormancy (e.g., Smith & Hallock, 1992) could be triggered by onset of photosynthesis by the symbionts. Understanding that process might provide insight into how food availability influences emergence from inactivity in both propagules and post-propagule individuals.

Dormancy in foraminifers provides mechanisms for the range expansion of species, recognized both in the fossil record and in modern species, including invasives. Weinmann et al. (2013) summarized the current latitudinal ranges of modern symbiont-bearing foraminifers, which are largely restricted to tropical and subtropical regions (between 30°N and 30°S), noting that foraminifers are primarily temperature limited and have extended ranges during times of increased temperatures at higher latitudes. Using Species Distribution Models, they predicted significant poleward expansion in the near future as the climate warms.

Langer et al. (2013) used the same modeling techniques, focusing specifically on *Amphistegina* species, predicting a 264 km southward range expansion for the species along the African coast by the year 2100. *Amphistegina* is a warm-water genus of particular interest because of its relatively wide temperature tolerances, with a range limited by the winter 13.7°C isotherm (Langer et al., 2012, and references therein). *Amphistegina lessonii* and *Am. lobifera* are Lessepsian invasives in the Mediterranean Sea, where they have been highly successful, both disrupting native foraminiferal biota (e.g., Langer et al., 2012) and altering coastal sedimentation (Triantaphyllou et al., 2009, 2012). Global range expansion could cause similar changes in other areas, and understanding what allows for range expansion is integral to accurate modelling and predictions.

Dormancy may play a role in the expansion of algal-symbiont-bearing foraminifers to higher latitudes during times of warmer climates, both in the future and in the geologic past. Although a warming climate may shift range-limiting isotherms to higher latitudes, the amount of sunlight available in winter should limit depth distributions. For instance, Langer et al. (2013) include both minimum chlorophyll-*a* content and maximum photosynthetically active radiation as biologically relevant predictor sets for their SDM models, both of which can be influenced by decreased light availability at higher latitudes. Smith & Hallock (1992) observed survival of *Ar. angulatus* and *Am. gibbosa* in extended periods of complete darkness, with recovery following a return to regular light exposure. Darkness-induced dormancy in symbiont-bearing foraminifers could allow winter survival of low-light adapted taxa in higher latitudes when increasing water temperatures allow for range expansion. If so, this could influence models predicting future range expansion, as well as interpretations of range expansion of symbiont-bearing foraminifers in the fossil record. For instance, Todd (1976) discussed the geographic limits of *Amphistegina* in the fossil record when climates were warmer. In the

Miocene, this genus occurred at latitudes as high as 50°N in the Vienna Basin and Poland, and to 37°S in Australia. In the Eocene, *Amphistegina* occurred as far north as 48°N on the Olympic Peninsula in Washington, USA, and as far south as 36°S in New Zealand. Photoperiodic dormancy is an adaptation that would favor symbiont-bearing foraminifers living at latitudes where light availability is highly seasonal.

Dormancy as a survival strategy in foraminifers also has implications for the interpretation of foraminiferal assemblages, which is important for paleoceanographic reconstructions (e.g., Kaiho, 1999; Kouwenhoeven et al., 1999; Sen Gupta & Platon, 2006), understanding the ecology of modern foraminiferal populations, and monitoring of environmental conditions. For instance, understanding the abundance of living infaunal taxa and the depth to which they can survive is key to understanding the ecology of living foraminiferal populations (e.g., Brooks, 1967; Buzas, 1977; Gooday, 1986; Corliss & Emerson, 1990; Ozarko et al., 1997; Saffert & Thomas, 1998; Berkeley et al., 2008).

The understanding of modern foraminiferal ecology also affects applications utilizing fossil foraminiferal assemblages, such as accurate paleoelevation reconstructions (e.g., Hayward et al., 2015), which rely on modern species analogues to understand paleoecology and accurately interpret changes in the assemblage with depth. If individuals capable of surviving burial via dormancy are mixed centimeters or tens of centimeters into the sediments, they may be stained by common techniques, though not actively living infaunally, thereby affecting the ecological and paleoceanographic implications of their presence. To effectively interpret assemblage patterns, it is necessary to understand the biology of foraminifers and how they react to changing conditions. As reviewed in this paper, evidence indicates that dormancy may be a common, but little recognized, facet of the foraminiferal life cycle.

SUGGESTIONS FOR FUTURE RESEARCH

Recognition of resistant propagules as common among the Foraminifera (Alve & Goldstein, 2003, 2010, and subsequent papers) has provided a fundamental breakthrough into understanding distributions of foraminifers, from local to biogeographical. The application of environmental molecular genetics to determine the diversity of propagules and the environments in which they can be found will help understand their importance in foraminiferal distribution. Possible questions are many: Are propagules of cosmopolitan taxa more likely to be found in ocean waters than propagules of endemic taxa? Can propagules of planktic taxa be found along specific density gradients in the pycnocline?

Given more limited recognition of post-propagule quiescence among foraminifers, the opportunities for exploration and application of the phenomenon are widespread. One can start with the basic "Who, what, why, and how?" Basic research to address "Who?" includes field, mesocosm and laboratory experiments to determine which taxa are capable of post-propagule dormancy and under what environmental conditions ("What and why").

To explore the mechanics of post-propagule dormancy (How?), approaches should include cytological, physiological, and chemical studies. Following the approach by which Goldstein (1997) recognized the fundamental nature of bi-flagellated gametogenesis in sexual reproduction in foraminifers, exploring taxa from different lineages is essential. Moreover, given the extreme diversity of the foraminifers, learning from both similarities and differences can provide additional insights.

Cytological studies are a necessity; cytological details should be compared between active and dormant foraminifers to understand the physical changes to the cell that occur with dormancy. Are there recognizable cytological changes in the shell or aperture (e.g., presence of an aperture plug) when an individual becomes dormant? Are there predictable cytological changes in dormant individuals over time? What cellular structures and organelles (or proportions thereof) are maintained and what deteriorates? In foraminifers with algal symbionts, how does the host cytoplasm change with time of inactivity and during emergence from inactivity, compared to the abundances and characteristic features of the algal symbionts both during and following inactivity?

Exploring physiological features associated with dormancy is equally essential. What are the triggering mechanisms for entry and exit from dormancy? What is the extent of metabolic suppression? How long can individuals survive in a dormant state? How do oxygen consumption and ATP expression, as indicators of metabolic activity, differ between active and dormant individuals? How does protein expression differ among active, newly dormant, prolonged dormant, and emerging specimens? Within taxa such as *Amphistegina* spp., which are capable of becoming dormant in response to multiple stressors, such as prolonged darkness, food availability, anoxia, and chemical exposure, are physiological responses to dormancy-inducing stressors similar or can fundamental differences be detected? In species hosting algal endosymbionts, how is the entrance and exit from dormancy coordinated between host and symbiont? These are but a few examples of possible avenues of research.

CONCLUSIONS

As this review shows, evidence from the literature for foraminiferal dormancy stretches back decades. Despite this, dormancy in the Foraminifera is little recognized and poorly understood, with much of the evidence interpretive at best. Yet the limited evidence available suggests that dormancy is far more ubiquitous in the foraminifera than previously realized. The literature indicates the presence of dormant stages in a wide variety of taxa, including those with diverse life strategies (both marine and freshwater benthic and marine planktic species). Thus, dormancy may be a key part of the life history of many, if not most, foraminifers.

The literature also demonstrates that a wide variety of dormant forms are present in the foraminifera, including uncovered resting stages, resistant resting cysts, and dormant juvenile propagules. Quiescent dormancy can be induced by a variety of environmental stresses, including temperature, starvation, estivation, anoxia, the presence of toxic chemicals, and aphotic conditions in the case of photosymbiotic species. This suggests that dormancy of some type may be a key survival strategy in the foraminifera, contributing to

their ability to react quickly and adapt successfully to changing environmental conditions.

Recognition of the potential for dormancy in the foraminifera can alter understanding of foraminiferal ecology. Foraminiferal populations, assemblages, and fossils are of fundamental importance in environmental, evolutionary, sedimentological, paleoenvironmental, micropaleontological, biostratigraphic and paleoceanographic research, as well as having many economic applications. Accurate interpretations of data in these fields often rely on an understanding of modern foraminiferal ecology. Given how ubiquitous dormancy seems to be, recognition of and inquiry into this basic biological process in the Foraminifera can provide breakthroughs in understanding the biology and ecology of these ubiquitous marine protists, and in turn influence understanding of foraminiferal records wherever they may be used.

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APPENDIX II

CHALLENGES IN USING CELLTRACKER GREEN ON FORAMINIFERS THAT HOST ALGAL ENDOSYMBIONTS

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Challenges in using CellTracker Green on foraminifers that host algal endosymbionts

Benjamin J. Ross and Pamela Hallock

College of Marine Science, University of South Florida, St. Petersburg, FL, United States of America

ABSTRACT

The uses of fluorescent microscopy and fluorescent probes, such as the metabolically activated probe CellTracker™ Green CMFDA (CTG), have become common in studies of living Foraminifera. This metabolic requirement, as well as the relatively quick production of the fluorescent reaction products, makes CTG a prime candidate for determining mortality in bioassay and other laboratory experiments. Previous work with the foraminifer *Amphistegina gibbosa*, which hosts diatom endosymbionts, has shown that the species is capable of surviving both acute chemical exposure and extended periods of total darkness by entering a low-activity dormant state. This paper explores the use of CTG and fluorescent microscopy to determine mortality in such experiments, as well as to explore the physiology of dormant foraminifers. The application of CTG was found to be complicated by the autofluorescence of the diatom symbionts, which masks the signal of the CTG, as well as by interactions between CTG and propylene glycol, a chemical of interest known to cause dormancy. These complications necessitated adapting methods from earlier studies using CTG. Here we present observations on CTG fluorescence and autofluorescence in *A. gibbosa* following both chemical exposure and periods of total darkness. While CTG can indicate vital activity in dormant foraminifers, complications include underestimates of total survival and recovery, and falsely indicating dead individuals as live due to rapid microbial colonization. Nonetheless, the brightness of the CTG signal in dormant individuals exposed to propylene glycol supports previously published results of survival patterns in *A. gibbosa*. Observations of CTG fluorescence in individuals kept for extended periods in aphotic conditions indicate uptake of CTG may begin within 30 min of exposure to light, suggesting darkness-induced dormancy and subsequent recovery can occur on short time scales. These results suggest that CTG accurately reflects changes associated with dormancy, and can be useful in laboratory experiments utilizing symbiont-bearing foraminifers.

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Corresponding author
Benjamin J. Ross,
benjaminross@mail.usf.edu

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INTRODUCTION

Fossil foraminiferal shells have been key tools in paleontological applications for more than a century. In the past half century, shell assemblages have also become widely used tools in environmental monitoring and assessment, and live foraminifers are increasingly being

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used in bioassay applications (i.e., *Alve, 1995*; *Yanko, Arnold & Parker, 1999*; *Frontalini & Coccioni, 2011*). Over the past 20+ years, foraminiferal assemblages and selected populations have become increasingly used to assess and monitor environmental conditions associated with coral reefs, which are in decline worldwide (e.g., *De'ath et al., 2012*; *Perry et al., 2013*; others). *Hallock (2000)* summarized the potential and benefits of using reef-dwelling larger benthic foraminifers (LBF) as indicators of water quality conducive to coral-reef accretion. Experimental approaches have included studies of growth rates (*Hallock, Forward & Hansen, 1986*), photosynthetic activity (e.g., *Talge & Hallock, 2003*; *Méndez-Ferrer, Hallock & Jones, 2018*), prevalence of morphological anomalies (e.g., *Prazeres, Uthicke & Pandolfi, 2016*), symbiont loss (e.g., *Hallock et al., 1995*), and, most recently, proteomics (e.g., *Prazeres et al., 2011*; *Stuhr et al., 2018*) and antioxidant capacity (i.e., *Prazeres, Uthicke & Pandolfi, 2016*; *Stuhr et al., 2017*).

Ross & Hallock (2014) explored the use of an LBF, *Amphistegina gibbosa* d'Orbigny, as a bioassay organism in toxicological studies relevant to coral reefs. During the development of protocols to determine the 48-hr 50% lethal concentration (LC50) of propylene glycol and 2-butoxyethanol (components of dispersants used in the Deepwater Horizon oil spill), we observed that *A. gibbosa* specimens were capable of surviving some level of toxic exposure by entering a dormant state, in which live individuals were visually indistinguishable from dead individuals, showing no signs of activity, which includes extension of the granuloreticulopodia, attachment to substrate, or production of visible waste (see *Ross & Hallock, 2016*, for further discussion and definition of dormancy in the Foraminifera). The identification of truly dead specimens required the use of a 72-hr recovery period to determine visually which individuals showed no evidence of activity. However, the uncertainty in the resulting LC50 estimates indicated the need for other readily applicable methods for determining mortality. Though seldom considered by researchers, dormancy is widespread among the Foraminifera (see *Ross & Hallock, 2016*, and references therein). Previous observations of dormancy in *A. gibbosa* under aphotic conditions (*Smith & Hallock, 1992*) reinforced the need to distinguish dormant from dead individuals and to better understand physiological facets of dormancy.

Fluorescence methods have a long history of use in recognizing live cells in a wide variety of cytological and histological applications (e.g., *Johnson et al., 1981*; *Taylor et al., 1986*; *Wommack et al., 1992*; *Patel et al., 2007*). The fluorescent probe, CellTracker™ Green CMFDA (CTG), is a non-terminal, non-fluorescent probe that, upon entering a living cell, can be cleaved by non-specific esterases common to living cells, producing a fluorescent compound, fluorescein, visible using a fluorescent microscope (functional mechanism summarized in *Bernhard et al., 2006*). The requirement of esterase activity means a cell must be alive to produce fluorescence.

How best to distinguish live specimens from dead shells in field samples and experimental treatments is an ongoing challenge and controversy among foraminiferal researchers (i.e., *Bernhard et al., 2006*; *Figueira et al., 2012*; *Frontalini et al., 2018*). *Bernhard et al. (2006)* compared the effectiveness of CTG with commonly used rose Bengal staining, which stains proteins and can stain dead cytoplasm as well as live. Bernhard and colleagues noted CTG's effectiveness specifically in identifying live foraminifers with transparent

shells, as well as organic-walled (allogromiid) foraminifers. *Figueira et al. (2012)* similarly showed the benefits of CTG over rose Bengal in identifying live salt-marsh taxa. These studies have generally been concerned with the efficacy of CTG in identifying live specimens from field collections (e.g., by incubating sediment cores).

The use of CTG in foraminiferal laboratory experiments, such as bioassays, is less established. *McIntyre-Wressnig et al. (2014)* employed it to identify live foraminifers for experiments and *Pucci et al. (2009)* used it to identify surviving individuals following experimental treatments. The primary objective of our study was to determine if CTG could be used to distinguish live from dead *A. gibbosa* in laboratory experiments. In several previous experimental studies, some specimens that visually appeared to be dead (i.e., normal symbiont color was highly altered and no rhizopodial activity was observed), regained normal golden-brown color and rhizopodial activity after placement in clean seawater with access to light (*Smith & Hallock, 1992*; *McCloskey, 2009*; *Ross & Hallock, 2014*). Our goal was determine to whether CTG could be used to determine activity in dormant foraminifers. The ability of CTG to persist post-fixation (common in methods using CTG on foraminifers, e.g., *Bernhard et al., 2006*) means that statistically robust numbers of specimens can be experimentally treated, then incubated in CTG, fixed, and observed at a later time. This efficiency, as well as the possibility of automation (e.g., image analysis technology) in place of human observations of vital activity, could greatly increase the potential for applications of *Amphistegina* spp. as bioassay organisms.

Objectives and strategy

The specific goals of this paper were the following: (a) to adapt methods utilizing CTG to observations of *A. gibbosa* in laboratory toxicity experiments; (b) to use these methods to determine if CTG fluorescence is a valid tool for distinguishing mortality versus survival in *A. gibbosa* that may be dormant, including both toxicity- and darkness-induced dormancy; and (c) to determine what fluorescence microscopy can reveal about the activity of dormant individuals.

To achieve these goals, preliminary experiments were required to address the challenge that some substances can interfere with CTG fluorescence. Then four experiments were conducted. Two examined vital activity of *A. gibbosa* exposed to different concentrations of PG for 48 hr. Experiment 1 included a 72-hr recovery period (as in *Ross & Hallock, 2014*), while Experiment 2 did not. These experiments were conducted to develop a protocol for using CTG in *A. gibbosa* bioassay experiments, and to compare results to determine whether CTG incubation immediately after exposure can indicate vitality in inactive, dormant individuals. Also of interest was whether CTG fluorescence and visually assessed "vital activity" (i.e., extension of granuloreticulopodia, attachment to the sides of well plates, and other visual indicators of life) following the 72-hr recovery period employed by *Ross & Hallock (2014)* indicated the same survival patterns.

The third experiment assessed vital activity in dormant *A. gibbosa* after 62 days in the dark in a temperature-controlled incubator, based on the results of *Smith & Hallock (1992)* and BJ Ross & P Hallock (2018, unpublished data) that found *A. gibbosa* can survive at least 20 months in total darkness in an apparently dormant state. The mechanisms of

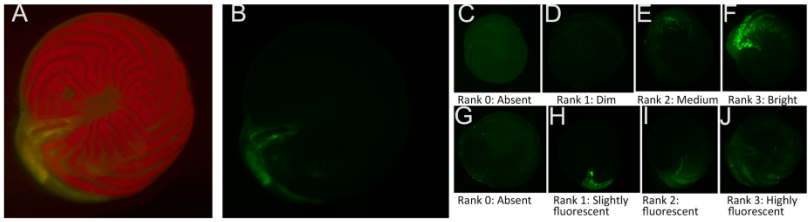

Figure 1 Ranking scale for CellTracker Green fluorescent brightness and coverage in *A. gibbosa*. (A) CellTrackerGreen and photosymbiont autofluorescence vs. (B) isolated CellTrackerGreen fluorescence; (C–F) fluorescence brightness ranking scale; (G–J) fluorescence coverage ranking scale.
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chemically- and dark-induced dormancy, and whether they are functionally the same, are unknown, so determining the effectiveness of CTG fluorescence under different dormancy conditions was of interest. In addition, the techniques developed for Experiments 1 and 2 could be applied without concern for interactions between propylene glycol and CTG.

The fourth experiment was carried out following observations in which high fluorescence was recorded in foraminifers that were visually identified as dead and which exhibited no subsequent recovery. This experiment was performed to determine whether microbial growth in dead *A. gibbosa* could affect the results of fluorescence experiments if individuals died during a 48-hr exposure, and whether this could explain this unexpected fluorescence.

METHODS

Preliminary experiments revealed two challenges in using CTG for these applications. The first challenge was with propylene glycol (PG), the chemical of interest in experiments by *Ross & Hallock (2014)* that established dose–response curves for *A. gibbosa*. When CTG was added to a PG-seawater treatment medium, no fluorescence was observed either in the media or in the foraminifers, even when PG was present in low concentrations and the foraminifers were active during incubation. This problem was addressed by thoroughly rinsing specimens exposed to PG before placing them in fresh seawater containing CTG and minimizing the length of the CTG incubation. An initial fluorescence presence-absence experiment exposing replicates of five healthy individuals to a range of incubation periods revealed that the shortest incubation period, 30 min, produced visible CTG fluorescence in all individuals; 30 min was thus used as the incubation period in all experiments, including non-PG experiments, for consistency.

The second challenge was because the diatom endosymbionts of *A. gibbosa* exhibit red autofluorescence, which can obscure CTG fluorescence in the endoplasm. As a consequence, CTG fluorescence is most visible in the outer chamber, where the ectoplasm is relatively free of symbionts. As seen in Figs. 1A–1B, even when using filters that exclude the red autofluorescence, CTG fluorescence can primarily be seen in regions of the endoplasm where symbionts are absent.

Challenges associated with autofluorescence were addressed by assessing responses using semi-quantitative ranking of fluorescence brightness and coverage. For fluorescence brightness (Fig. 1B), a ranking of 0 indicates absence of fluorescence; 1 is described as dim fluorescence; 2 as medium fluorescence, easily discerned but not bright; and 3 as bright, often scintillating fluorescence. For fluorescence coverage (Fig. 1C), a ranking of 0 indicates absence of fluorescence; 1 is described as isolated or spot fluorescence, with up to five individual fluorescent spots visible, but not in any obvious distributional relationship; 2 as low coverage (typically in the youngest chamber), with <25% of observable surface area fluorescing green; 3 as medium coverage, between 25% and 70% of the surface area fluorescent; and rank 4 as high coverage, with >70% of the surface area fluorescing green. Rank 1 coverage was generally determined to be the result of contamination or epibiotic growth and for data analysis was combined with rank 0 as representing no fluorescence within the cell.

All experiments were conducted in unfiltered oceanic seawater of salinity ~38 collected from the sample sites at Tennessee Reef in the Florida Keys (24.7523°, −80.7549–80.7549°), at 25 °C and under a 12-hr dark/12-hr light illumination cycle, with light levels of ~10 μmol photons/m^2/s (sufficient for growth and life activity in *A. gibbosa*, without causing photo-oxidative stress; *Hallock, Forward & Hansen, 1986*; *Talge & Hallock, 2003*) measured using a LI-COR photometric sensor; the exception to the dark/light cycle was the 62-day darkness treatment.

Experimental methods

Experiments 1 and 2 applied four experimental treatments, based on results from *Ross & Hallock (2014)*: (a) control concentration (0% PG media); (b) a low concentration of 1.5% (v/v) PG, observed to not cause mortality or initiate dormancy in any foraminifers; (c) an intermediate concentration of 3% PG, observed to cause 100% apparent mortality (lack of rhizopodial activity and waste production) in the experimental specimens following the 48-hr exposure, but exhibiting 100% recovery by 72 hr after removal from PG exposure; and (d) a high concentration of 8% PG, observed to cause 100% apparent mortality and no recovery by 72 hr after removal from PG exposure. In Experiment 2, the high concentration was raised to 10% because a few individuals exposed to 8% PG showed signs of recovery following the first experiment's 72-hr recovery period. Each experimental treatment included five replicates, and each replicate included five *A. gibbosa* specimens.

For the first PG experiment, the foraminifers were exposed to treatment conditions for 48 hr. Specimens were rinsed with clean seawater three times to remove residual PG, then incubated in 0.3 μM CTG in unfiltered seawater at a salinity ~38 for 30 min at ~25 °C. After incubation, the foraminifers were again rinsed three times with clean seawater to remove any remaining CTG, so that uptake would not occur after the 30-min incubation and reflect later recovery. Preliminary experiments revealed that these procedures produced observable CTG fluorescence, while hypothetically limiting recovery of metabolic activity of the foraminifers following removal from a treatment. The foraminifers were then allowed to recover for 72 hr in clean seawater, following the protocols developed by *Ross & Hallock (2014)*. After 72 hr, color and activity of each specimen were visually assessed,

then individuals were imaged while living to record presence of fluorescence (as described below in the 'Imaging and Statistical Analysis' section).

For Experiment 2, 48-hr duration treatments again were conducted, with the 8% concentration of PG replaced by 10%. In addition, a "dead" control treatment was included to test the hypothesis that dead individuals would exhibit no fluorescence. Specimens were killed by placing the foraminifers in deionized water for the duration of the 48-hr incubation. The foraminifers were rinsed and exposed to CTG using the same procedures as in Experiment 1, but without a 72-hr recovery period. Instead, digital photographs were taken immediately after they were rinsed to remove the CTG. Color and activity of each specimen were visually assessed, then individuals were imaged while living to record the presence of fluorescence.

For Experiment 3, the extended darkness treatments, the foraminifers were kept in replicates of 5 ml microcentrifuge tubes with pinholes pierced at both ends to allow for gas exchange. These tubes were kept in semi-opaque containers, double wrapped in aluminum foil, in a temperature-controlled incubator at 25 °C. After 62 days in darkness, one treatment of five replicates of five specimens each was incubated in CTG for 30 min in darkness. The other was incubated in CTG for 30 min in ambient room light, which is sufficient to allow survival and growth in *A. gibbosa* kept in aquaria with no other light sources. The foraminifers were then photographed live under an epifluorescent microscope.

For Experiment 4, specimens were divided into two treatments of 25 specimens, each placed in seawater in a large, sealed Nalgene bottle, and then killed by exposure to temperatures of 60–65 °C for approximately 4 hr. On removal, the specimens were transferred to well plates filled with seawater for 48 hr before incubation in CTG and imaging. One treatment was kept in seawater from the heat-treated Nalgene throughout the experiment, including during CTG incubation; this seawater was assumed to be relatively sterile. The other treatment received new, untreated seawater after heat treatment. Specimens were individually assessed visually for mortality before and after live fluorescence imaging.

Imaging and statistical analysis

All images were taken using a Leica MZ FLIII epifluorescent stereomicroscope with FLUOIII filter system. The CTG was excited using a mercury short-arc lamp, and filtered to a range compatible with GFP (green); a Leica Filter cube N3 (Ex546/12 & Em 600/40 with a 565 beam splitter) was used. A standard magnification of 40× was used and the microscope was focused on the foraminifer's shell using reflected light before fluorescence imaging to standardize focal depth. Standard color images were taken first, then green epifluorescent images (to visualize CTG), followed by combined RGB epifluorescent images (to visualize combined CTG and endosymbiont autofluorescence) using 30 s exposures. Resulting fluorescence was ranked according to scales for both brightness and coverage (Fig. 1). Foraminifers were not fixed, but photographed while living. This was initially done so that they could be observed for recovery of activity following photography. Although they were found to suffer from photic shock post-photography, making this goal unreliable

(see Discussion), in later experiments we continued to photograph specimens live and unfixed for consistency across treatments.

Statistical tests were performed using MATLAB with the Fathom toolbox (*Jones, 2015*). Because distributions were non-normal, all tests performed were non-parametric. Distributions of resulting fluorescence in each experiment were compared using Mann–Whitney tests, single- or two-factor non-parametric Analysis of Variance tests, and non-parametric Analysis of Covariance tests depending on comparisons being made. All tests were considered significant at $p = 0.05$. Error bars in figures represent standard errors of means.

RESULTS

Of the two response parameters used in this study, fluorescent coverage and fluorescent brightness, the latter was generally more useful. Based on non-parametric ANOVA, significant differences in coverage were only observed in Experiment 1 (72-hr recovery after PG exposure, $df = 92$, $p = 0.03$) between the 1.5% PG and control treatments ($p = 0.02$), 3% PG treatment (0.01) and 8% PG treatment (0.01). No significant difference in coverage was observed in Experiment 2 (assessed immediately after PG exposure, $df = 96$, $p = 0.6$).

Significant differences were seen in fluorescent brightness of specimens among PG treatments in both experiments. In Experiment 1, non-parametric ANOVA showed an overall significant difference ($p = 0.003$) and pairwise comparisons showed differences between the 8% propylene glycol treatment and the control ($p = 0.02$), 1.5% PG (0.001) and 3% PG (0.015). In Experiment 2, a significant difference was observed ($p = 0.001$) between the 3% PG treatment and the control (0.002), 1.5% PG (0.004) and 10% PG ($p = 0.001$) treatments, as well as between the 10% PG treatment and the 1.5% PG treatment (0.004). Although the 10% PG treatment did not differ significantly in brightness or coverage from the control, specimens showed no visual signs of recovery, consistent with previous experiments.

Comparing the two PG-exposure experiments using a 2-factor ANOVA ($df = 192$), Experiment 2 treatments (no recovery period) showed significantly higher fluorescent brightness (Fig. 2C) and significantly lower coverage (Fig. 2D) than Experiment 1 treatments (factor 1 (recovery vs. no recovery), $p = 1 \times 10^{-3}$; factor 2 (PG%), $p = 1 \times 10^{-3}$; factor 1x2, $p = 1$ for both analyses).

Comparing visually determined vitality with that indicated by the presence of fluorescence using a non-parametric ANCOVA test of average "live" individuals per day (either visually identified as living or by fluorescence presence) indicated a significant difference ($p = 0.006$, $df = 32$) between the measures for Experiment 1, with follow-up pairwise Mann–Whitney tests indicating significantly fewer specimens exhibited fluorescence than visible activity in the control treatment ($p = 0.03$). For the other treatments, the differences were not significant (Fig. 3). In Experiment 2, the NP-ANCOVA indicated a significant difference ($p = 0.001$, $df = 32$) in average "live" individuals per day with no recovery after PG exposure. Follow up pairwise tests showed that fluorescence indicated significantly more live individuals than visual assessment in the 3% ($p = 0.008$)

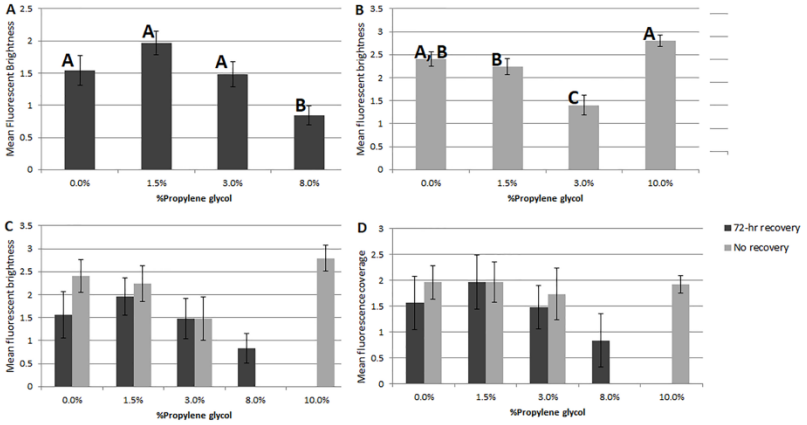

Figure 2 Comparisons of fluorescent brightness and coverage in *A. gibbosa* following 48-hr propylene glycol exposure with and without 72-hr recovery period. (A) Experiment 1–mean rankings in CTG fluorescent brightness following 48-hr propylene glycol exposure and 72-hr recovery in seawater. Letters above the bars indicate that results that are statistically similar; error bars represent standard errors of means; (B) Experiment 2–distribution and significant differences in CTG fluorescent brightness following 48-hr PG exposure with no recovery period; (C) comparison of rankings of fluorescent brightness following 48-hr PG exposure and 72-hr (Exp. 1) or no (Exp. 2) recovery; (D) comparison of rankings of fluorescent coverage following 48-hr PG exposure and 72-hr (Exp. 1) or no (Exp. 2) recovery.

Full-size DOI: 10.7717/peerj.5304/fig-2

and 10% ($p = 0.008$) treatments (Fig. 3). In Experiment 3, using specimens held in the dark for 62 days, a Mann–Whitney test ($df = 19$) showed no significant difference in fluorescent coverage between specimens incubated in CTG in the dark and those incubated in CTG in the light. However, specimens in the treatment incubated in the light showed significantly higher fluorescent brightness than those incubated in the dark ($p = 0.002$) (Fig. 4). Non-parametric 1-way ANOVA comparisons of specimens from the light-incubated and dark-incubated treatments of Experiment 3 to the control treatment of Experiment 1 ($df = 67$) indicated a significant difference in both brightness ($p = 0.001$) and coverage ($p = 0.001$). Follow-up pairwise comparisons showed significantly higher brightness in the control than the dark incubated specimens ($p = 0.001$), and significantly lower fluorescent coverage between control and both dark incubated ($p = 0.006$) and light incubated ($p = 0.001$) specimens. Observations of symbiont autofluorescence (Fig. 5) indicated a concentration of symbionts towards the center of the foraminifers following the extended dark treatment, with presence throughout the shell reestablished within 99 h.

In the microbial growth experiment (Exp. 4), a Mann–Whitney test showed no significant difference in brightness ranking ($df = 22$) between the heat-killed and new seawater treatments (Fig. 6), but the new seawater treatment showed significantly higher fluorescence coverage ($p = 0.009$).

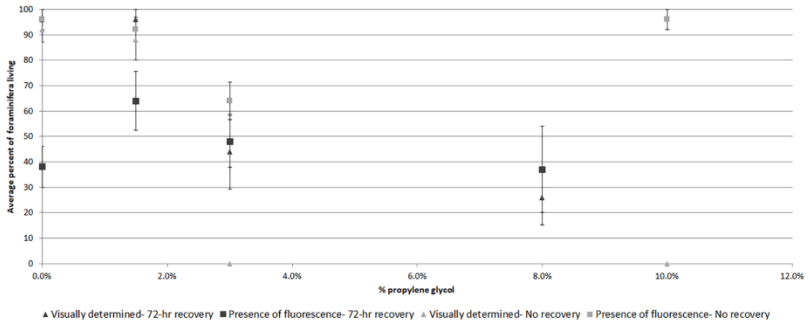

Figure 3 Average percent of foraminifera living after 48-hr exposure to propylene glycol with and without 72-hr recovery period as determined visually and by presence of CTG fluorescence. Average survival as determined by visible signs of activity or by presence of fluorescence following a 72-hr recovery period from PG exposure (Exp. 1) and no recovery from PG exposure (Exp. 2). Error bars represent standard errors of means.

Full-size DOI: 10.7717/peerj.5304/fig-3

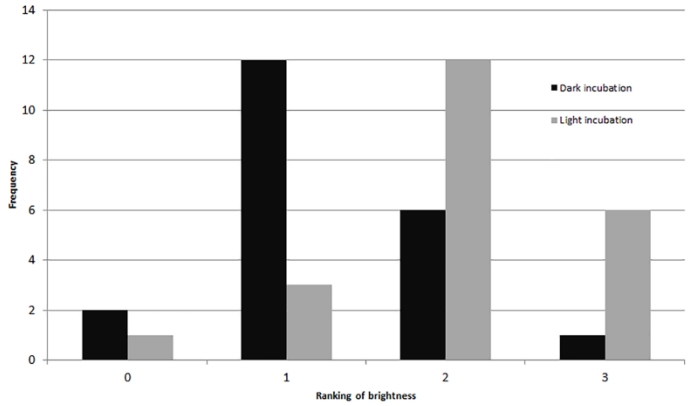

Figure 4 Fluorescent brightness in *A. gibbosa* following 62 days in aphotic conditions. Histogram comparing fluorescent brightness in *A. gibbosa* held in the dark for 62 days, then incubated in CTG in either complete darkness or light (Exp. 3). Frequency includes all specimens observed.

Full-size DOI: 10.7717/peerj.5304/fig-4

DISCUSSION

Challenges and strategies

The first goal of this research was to adapt methods utilizing CTG to determine survival of *A. gibbosa* in laboratory toxicity experiments. This objective was motivated by complications associated with dormancy that were observed during bioassay experiments (*Ross & Hallock, 2014*). Can fluorescence be used to quickly distinguish living from dead *A. gibbosa* in

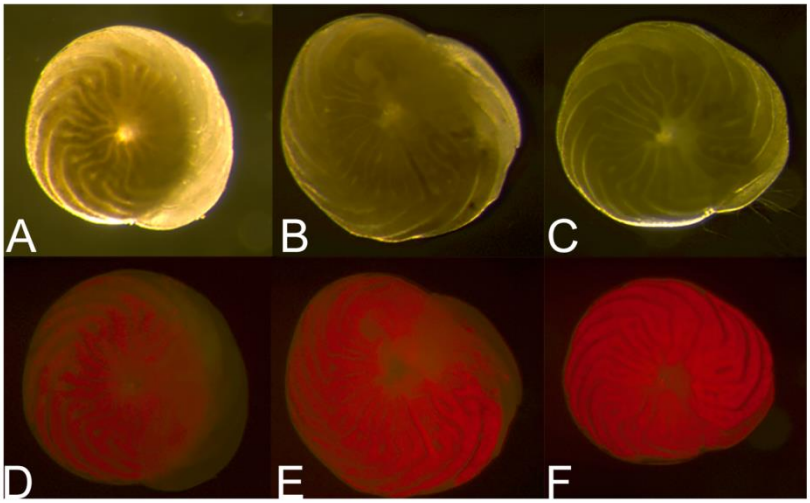

Figure 5 Symbiont recovery in *A. gibbosa* following 62 days in aphotic conditions. Light microscope (A–C) and fluorescent microscope (D–F) images of (A) and (D) 0 h, (B) and (E) 25 h and (C) and (F) 99 h of symbiont population recovery following 62 days in darkness (Exp. 3).

Full-size DOI: 10.7717/peerj.5304/fig-5

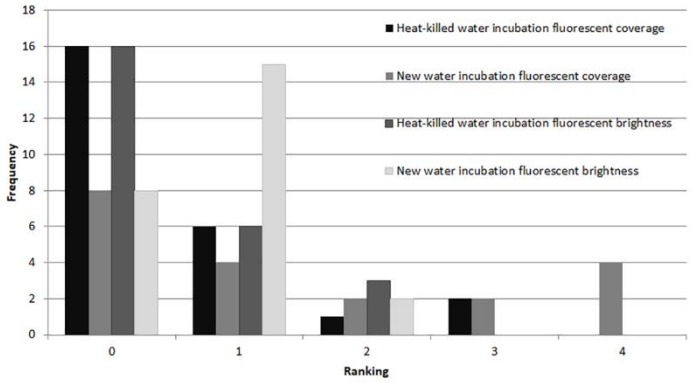

Figure 6 Fluorescence in heat killed *A. gibbosa*. Histogram comparing ranked fluorescent coverage and brightness in heat-killed *A. gibbosa* incubated in either the original heat-killed or new seawater (Exp. 4). Frequency includes all specimens observed.

Full-size DOI: 10.7717/peerj.5304/fig-6

experimental studies? This goal was complicated by several factors that required preliminary experiments to find workable experimental protocols.

The primary impediment to quantitative observations of fluorescence in *A. gibbosa* is autofluorescence of the diatom symbionts. Hypothetically, given the consistency of the mortality pattern in previous work (*Ross, 2012*; *Ross & Hallock, 2014*), fluorescent brightness and coverage should show a consistent dose/response, since activation of CTG is linearly associated with active metabolism. However, differences in fluorescence among PG treatments were minimized by the masking effect of the symbionts (Fig. 1). These challenges prompted the use of a simple presence/absence scale of survival/mortality and the use of ranking to assess fluorescence. While this solution introduced some possibility of bias, one of the most valuable roles of foraminifers in environmental indicator work is use as a rapid, low-cost assay and bioindicator taxa. Because of the size and abundance of the shallower-dwelling species of *Amphistegina*, which occur in subtropical/tropical coastal environments nearly worldwide, qualitative measures such as visual "bleaching rank" (e.g., *Hallock et al., 2006*, and references therein) and visual assessment of rhizopodial activity to determine vitality (e.g., *Ross & Hallock, 2014*) have proven to be widely applicable. Because use of CTG requires specialized microscopy equipment, now that challenges regarding its use have been identified, further development or adaptation of these methods can include the use of quantitative image-analysis techniques, though the shape and reflective nature of the *Amphistegina* shell impose challenges to such analyses.

Suppression of CTG-induced fluorescence by PG was the second challenge that had to be solved. Solving this problem necessitated removal of experimental specimens from the treatment medium and repeated rinsing in fresh seawater before incubation in CTG. The rinsing process added another complication. Since the rate of recovery of *A. gibbosa* from chemically-induced dormancy was not known, minimizing the opportunity for specimens to recover was important to assess the presence of metabolism under dormancy, instead of during recovery. Thus, we used preliminary experiments to determine a CTG incubation period that was as short as possible, which was 30 min. Because the size of *A. gibbosa* and the short incubation period undoubtedly limited penetration by CTG, we directly exposed specimens to the 0.3 μM CTG-seawater solution in small well plates. Our CTG concentrations were higher and incubation times much shorter than previous applications (e.g., *Bernhard et al., 2006*; *Pucci et al., 2009*), where full sediment cores were incubated in 0.1 μM CTG solution for much longer periods (at least 6 h), then fixed, and CTG fluorescence was used to determine which specimens in sampled sediment layers were alive at the time of sampling. We used the preliminary experiments to adapt and employ strategies to provide consistency across our experiments and to meet our objectives, which were much different from those of previous studies.

Another challenge we encountered was photic damage associated with the high-energy excitation wavelengths required to produce fluorescence. For instance, most individuals exhibited some fluorescence following 62 days in the dark, consistent with previous observations by *Smith & Hallock (1992)* of recovery after 12 months in total darkness. However, experimental specimens were very sensitive to light and although activity resumed during the 72-hr recovery, symptoms of photic damage (i.e., loss of color, or

"bleaching") were subsequently observed. Photic damage during imaging also complicated observations of the individuals in the PG treatments, as these individuals also showed signs of photic stress. One of our goals was to compare results from CTG treatment with post-recovery visual assessments by observing vital activity of the foraminiferal specimens days to weeks after removal from experimental treatment. Although specimens were repeatedly examined for up to 7 days, photic damage complicated assessing the chronic influence of the chemical treatments over longer time scales. Thus, future studies with such goals will require more sets of specimens so that assessment of longer term recovery can utilize specimens that have not been previously exposed to damaging photic stress.

Microbially-induced fluorescence

A major motivation for the use of CTG versus rose Bengal in studies that assess the assemblages of foraminifers alive in sediments when sampled is to avoid the ambiguities associated with rose Bengal. This protein stain can stain cytoplasm and microbes in dead shells (e.g., *Bernhard et al., 2006*). As became clear in Experiments 2 and 4, microbial growth also can produce significant fluorescence in CTG-treated dead specimens. Some fluorescence was observed in nearly half (48%) of the specimens maintained in non-sterile seawater medium for 48 hr after being heat killed (Fig. 6).

The fluorescence in the heat-killed treatments was relatively dim compared to the bright fluorescence seen in the 10% PG-exposure treatment. Many aerobic bacteria can utilize PG as a growth medium in concentrations of 10% or less (e.g., *Lee et al., 2003*, and references therein), and PG as a contaminant is known to be readily biodegraded by microbes (e.g., *Biró et al., 2014*, and references therein). In Experiment 2, if the microbial assemblage in the PG-killed foraminifers was able to bloom within the shell, feeding on the combination of PG and the degrading cytoplasm of the host and associated algae, that microbial growth could be responsible for the substantial fluorescence observed in specimens in the 10% PG treatment. Microbially-induced fluorescence may have influenced the results from other treatments; however, since all other treatments (including the 8% PG treatment in Exp. 1) showed recovery in some individuals as determined by visible activity, the influence is less clear. In future bioassay work with chemical toxicity, the influence of the chemical on microbial growth should be considered.

Determination of mortality

A major motivation and second objective for this study was to determine if the CTG fluorescence probe could aid in distinguishing mortality from dormancy in *Amphistegina*, primarily as an aid in bioassay experiments. Other applications include determining survival potential in aphotic conditions, such as burial in sediment. Unfortunately, the results did not establish a definitive relationship between survival determined visually post recovery and esterase activity as indicated by fluorescence. Instead, many of the findings seemed counterintuitive or even contradictory, suggesting a complex relationship between stress, dormancy, survival, and fluorescence.

Even with specimens known to be dead, fluorescence ranged from none to brightest, depending upon incubation media. For example, specimens killed by treatment in deionized

water (Exp. 2) showed no fluorescence, which would be expected in dead foraminifers in which there was no microbial activity. Yet heat-killed specimens (Exp. 4) kept in seawater for 48 hrs exhibited fluorescence, especially in the untreated seawater (Fig. 6). Paradoxically, fluorescence in individuals in the 48-hr 10% PG-exposure treatment with no recovery (Exp. 2) was not significantly different from controls and was significantly brighter than the 3% treatment specimens (Fig. 3), despite exposure being above the threshold previously observed to kill 100% of the experimental specimens (*Ross, 2012*; *Ross & Hallock, 2014*) and despite those individuals showing no visual evidence of survival. The highest concentration treatment in Experiment 1, 8% propylene glycol with a 72-hr recovery period, exhibited the least bright fluorescence in the experiment, and was significantly different from all other treatment concentrations.

Specimens in the 3% PG-exposure treatments provided interesting results indicating that fluorescence presence can sometimes be a better indicator of survival than visual signs. In the no-recovery treatment (Exp. 2, Fig. 3), none of the specimens exhibited visual signs of activity at the end of the 48-hr treatment, while 64% exhibited fluorescence. This percentage was similar to the ~50% survival for the 3% PG treatment after 72-hr recovery (Exp. 1, Fig. 3). Specimens from the latter treatment were not significantly different from controls in rankings in either fluorescent coverage or brightness. In comparison, specimens in the 1.5% PG treatments with and without recovery (Exp.1 and 2) were not significantly different from control specimens.

However, specimens in the 3% PG-exposure treatment with no recovery (Exp. 2), which were likely dormant, were significantly less bright than in the other treatments (Fig. 2B), which could indicate either metabolic depression, decreased enzymatic activity, or limited uptake of media in response to PG exposure that continued during the 30 min CTG incubation. The fluorescence in some individuals was sufficiently dim to be categorized as absent, or CTG may have been excluded entirely, leading to an underestimation of survival. If the specimens in the 10% PG treatment died before they could react to the presence of PG by becoming dormant, CTG may have penetrated the shell and been taken up by the microbes throughout the dead foraminiferal specimens. This could be true in other treatments as well, leading to an overestimation of survival in treatments where mortality occurred.

An initial goal of this paper was also to compare CTG with post-recovery visual assessments to directly compare the mortality rates from each method. Unfortunately, *A. gibbosa* is light sensitive, and photic damage associated with the high-energy excitation wavelengths required to produce fluorescence, and the exposure time necessary to record it, damaged the specimens. This photic damage made visual assessment of vitality and subsequent comparisons to CTG results, unreliable.

Behavior under dormancy

The final question motivating this study was whether use of CTG could provide insight into the behavior of *A. gibbosa* under stress, specifically in dormant states. The results offer some insights while raising more questions.

Firstly, the results suggest that dormancy suppresses metabolism and is related to survival under stress. Based on the results of the microbial-growth experiment, the differences between visual assessment of survival and presence of fluorescence (Fig. 3) becomes easier to interpret. In the control and 1.5% PG treatments, there was no or little PG to affect the foraminifers, so fluorescence presence and visually assessed survival were both near 100%. In the 3% PG treatment without recovery, the presence of fluorescence indicated much higher survival than visual assessment, though the reduction in fluorescence brightness was significant (Fig. 2B). These observations are consistent with previous observations that exposure to a 3% concentration of PG can trigger dormancy (*Ross & Hallock, 2014*). The 10% concentration killed the specimens, either before or in spite of any defensive reactions, and microbial growth quickly colonized the new food sources, leading to no significant difference from control specimens in the presence of fluorescence, while showing no visual signs of vitality (i.e., rhizopodial activity).

The results also suggest that recovery from dormancy can be very fast once removed from the stressor. In the darkness experiment (Exp. 3), a significant difference between the control (Exp. 1) and the dark-incubated treatment supports the hypothesis of metabolic depression in dormant individuals. The significantly higher brightness in the CTG treatment incubated in light, and its non-significant difference from the control, suggests that metabolic recovery may begin within 30 min (the length of incubation). This result suggests that any experiments involving dark-adaptation of *A. gibbosa* must be performed in darkroom conditions.

More importantly, the results of Experiment 3 indicate that recovery from darkness-induced dormancy can occur sufficiently rapidly to make metabolic depression or dormancy effective on short time scales, possibly overnight. Although research has largely focused on the photo-toxic effects of increased light levels in photosymbiotic foraminifers (i.e., *Hallock, 2000*), *Prazeres, Uthicke & Pandolfi (2016)* showed decreased antioxidant levels in *Amphistegina lobifera* exposed to low light conditions as well. *Prazeres, Roberts & Pandolfi (2017)* also observed that exposure to elevated temperatures and nitrate levels reduced survivorship and fecundity of *A. lobifera*, which were exacerbated by below optimal light levels. These observations demonstrate the negative impacts low- or absent light can have on LBFs, especially in warming and increasingly more nutrient-rich coastal waters, and support the hypothesis that dormancy in the absence of light may be a survival mechanism in response to physiological stress.

As ocean conditions warm, *Amphistegina* species worldwide have been observed to move poleward, and models estimate that they will continue to do so (*Langer et al., 2013*). Similarly, they have been found poleward of their current distributions in the geologic record, as far as 50°N and 37°S in the Miocene (*Todd, 1976*). *Schmidt et al. (2015)* found that another symbiont-bearing foraminifer, *Pararotalia* sp., which has invaded the eastern Mediterranean as temperatures have warmed, demonstrates less cold-tolerance than native species. This suggests that species undergoing temperature-mediated expansion are not necessarily adapting to local conditions. If the same is true concerning light levels, the ability to enter darkness-induced dormancy on short time scales would be a valuable adaptation for tropical foraminifers expanding into areas where light availability substantially varies seasonally.

Comparing patterns of fluorescence brightness and coverage in specimens before and after a 72-hr recovery from PG exposure (Fig. 3) also provides hints of how *A. gibbosa* responds to toxic chemicals, as well as to the behavior of CTG within a foraminiferal cell. The lack of significant differences in fluorescence coverage among treatments post-recovery (Exp. 1), as well as the significant decrease in brightness and visible coverage between no-recovery (Exp. 2) and 72-hr recovery (Exp. 1) experiments, indicates that the CTG signal diminished over the recovery period. In the no-recovery experiment, CTG-induced fluorescence was largely visible in the large final chamber, in which the aperture is located and where most uptake occurs from the environment. This chamber typically houses ectoplasm that gives rise to the reticulopodia and is largely symbiont free. This chamber makes up a considerable proportion of the visible surface area, influencing estimates of fluorescence coverage. The brightness differences likely are also related to initial uptake, with most of the fluorescence concentrated in a smaller area devoid of symbionts, leading to a brighter local signal with no autofluorescent interference. The decrease in brightness over 72 h may reflect dispersal of CTG through the cytoplasm over time.

The concentration of CTG fluorescence in the outer chambers was observable in all treatments of Experiment 2, in which specimens were taken from the PG exposure, rinsed and immediately exposed to CTG. Similar concentration was not seen in the 72-hr recovery experiment (Exp. 1), the darkness experiment (Exp. 3), or the microbial growth experiment (Exp. 4). Since CTG was observed in the final chambers of the control specimens, the uptake likely reflects how foraminifers take in material from outside the shell. The fluorescence in Exp. 2 also appeared more granular than in other experiments, possibly because the CTG was still concentrated in the ectoplasm of the final chamber instead of dispersed through the endoplasm in the shell interior. The absence of concentrated fluorescence in the ectoplasm after recovery could be explained by its integration and diffusion during the recovery period. In the microbial growth experiments (Exp. 4), because the foraminiferal cell was dead, CTG entered the shell via passive diffusion. Further development of these methods may necessitate either a longer incubation in CTG, or a recovery period less than 72 hr to allow for observations of activity deeper in the cells. The question of why the fluorescence has such a granular appearance in the outer chambers is one that may be answerable by light or transmission electron microscopy. Granularity was also observed in specimens in the 10% PG treatment, where no recovery of ectoplasmic activity was observed, indicating uptake of CTG by live microbes could occur in the outer chamber.

In the 10% PG treatment (Exp. 2), specimens showed no recovery and were presumed to have died sometime during the initial PG exposure and not during post-CTG incubation. The concentration of fluorescence in the newest chamber in this treatment provides clues that the outer chamber can limit entry of toxic substances into the cell interior. That the CTG wasn't able to diffuse more fully into the cell, despite the ectoplasm being inactive, suggests there may be a physical barrier that impedes exchange between the outer and inner chambers. This could be a matter of time, but other treatments showed more complete penetration. This hypothesis is supported by results showing the same concentration in the outer chambers of the dormant 3% PG treatment specimens, which suggests this barrier

is established before metabolic activity is reduced. If present, it may be visible via TEM cytological analysis as an electron-dense body in the foramin of the penultimate chamber.

Following PG exposure and a 72-hr recovery period (Exp. 1), the 1.5% PG treatment exhibited increased brightness, while the control and 3% treatments showed similar brightness and the 8% treatment showed reduced brightness (Fig. 2A). This pattern is similar to that seen by *Tzovenis et al. (2004)* in a study of the effects of PG on the chlorophyte alga *Dunaliella tertiolecta*, where exposure to low concentrations led to an increase in activity (measured by growth). *Tzovenis et al. (2004)* suggested that PG may play the role of a micronutrient at low concentrations; the increased activity of recovered *A. gibbosa* observed here (as measured by the brightness of fluorescence) could indicate a similar effect, and support the hypothesis that the non-dormant specimens in the 1.5% treatment are actively taking in the PG in the media, or feeding on the bacteria that fed on the PG.

Questions for further research

The results of our study raise a number of important questions about *A. gibbosa* and its reactions to stress. An obvious question raised by observations of darkness-induced dormancy is: what happens to the photosymbionts? Figure 5 shows the difference in light microscope coloration and symbiont autofluorescence in specimens of *A. gibbosa* observed at different times after removal from 62 days in aphotic conditions. Full recovery of color required approximately four days, although observations by *Smith & Hallock (1992)* indicate recovery can differ depending on length of time in darkness. Both imaging techniques showed a reduction in symbiont-related color, and the symbionts present when newly removed from darkness seemed to be concentrated deeper in the cell near the center, with the population dispersing throughout the cell over time. What is unclear is whether this concentration is due to movement of the symbiont population; shrinkage of the host cell; digestion of some of the total symbiont population; or a combination of the above.

As dormancy is well known in diatoms (i.e., *Jewson, Rippey & Gilmore, 1981*; *Sicko-Goad, Stoermer & Kociolek, 1989*; *Itakura, Imai & Itoh, 1997*; *Ribeiro et al., 2011*), a possible interpretation is that a small symbiont population survives, also in a dormant state, concentrated deeper in the cell when there is no light available for photosynthesis, possibly as a way for the host to safely control recovery of the symbiont population. When light becomes available, the diatoms likely asexually reproduce resulting in the observed lag between removal from darkness and recovery of healthy foraminiferal color. What is known from previous research is that the diatom-symbiont population can apparently outlive the host. When *A. gibbosa* was kept in darkness for 18 months, then reintroduced to the light, some specimens showed recovery of color, but no resumption of host vital activities (*Smith & Hallock, 1992*). Cytological analyses using light or transmission electron microscopy may clarify the relationship between host and symbiont in this dormant state, as well as in toxin-induced dormancy, where obvious color loss is not as easily explained. How the relationship between the host and symbionts changes on the short and long term is important to understanding how *A. gibbosa* survives environmental stress.

Another important question is: What is happening to the foraminiferal cytoplasm when dormant, either chemically or darkness induced? As discussed above, the pattern of CTG uptake observed in Experiment 2 could be explained by the presence of a barrier in the foramin between the final chamber and the penultimate chamber. If present, such a barrier might be visible via cytological analysis. Similarly, cytological analysis of individuals in long-term darkness induced dormancy can provide insight into whether the host is digesting cytoplasm or symbionts.

Most of these questions also apply to what is happening in the foraminifers when dormancy is initiated and during recovery. Cytological and metabolic studies are essential to understanding physical changes. Proteomics could also be very informative in understanding the proteins involved during initiation, dormancy, and exit from dormancy. Identifying the proteins involved might provide insight into the evolutionary origins of dormancy, and to identify the potential for dormancy in foraminiferal taxa in which it has not been directly observed.

CONCLUSIONS

Fluorescence has the potential to be used in studies involving foraminiferal mortality, but more research is needed to confirm the relative accuracy of identification methods and to determine the ideal incubation time to allow CTG to fully enter the cell. For bioassay applications, some dormant individuals can be recognized as live without the necessity of a recovery period, but percent survival will be underestimated. In studies of live foraminiferal assemblages from stressful environments, the rapid colonization of dead shells by microbes suggests the possibility of overestimation due to the presence of recently dead individuals, while failing to identify live, dormant individuals.

By masking the signal, autofluorescence complicates the application of CTG for foraminifers that host algal endosymbionts. Because relatively little is known about the relationship between host and symbiont in stressful conditions, especially under extended darkness, other approaches such as observations of internal ultrastructure will be required. Observations of symbiont autofluorescence itself, though, may have interesting implications for a number of applications that involve visual observation of symbiont-bearing foraminifers (e.g., for signs of photic stress).

When the influence of microbial growth is considered, observed patterns of fluorescence support previous interpretations of the effect of propylene glycol on *A. gibbosa*: chronic low-level exposure at low concentrations, dormancy and metabolic depression above a certain threshold, and mortality at higher concentrations. These patterns also support the hypothesis of some kind of barrier between the outer and inner chambers, such as the formation of an "apertural plug".

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Benjamin J. Ross conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Pamela Hallock conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Foraminiferal samples were collected under the Florida Keys National Marine Sanctuary Permit FKNMS-2015-026.

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5304#supplemental-information.

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APPENDIX III

TRANSMISSION ELECTRON MICROSCOPY METHODS

Introduction

In addition to the work presented in the dissertation itself, I spent a significant amount of time attempting to successfully prepare *Amphistegina gibbosa* for Transmission Electron Microscopy (TEM) analysis, in order to examine ultrastructural changes associated with dormancy. Initially, I had planned to use methods developed by Talge & Hallock (1995, 2003). However, gaps in the methods as recorded in these publications caused initial difficulties, and I was not able to successfully replicate the quality of fixation. I tried a number of different methods drawn from both the literature and personal communications, largely involving changes in initial fixatives, but was not able to fix any specimens to my satisfaction. Although the diatom symbionts preserved well, with visible chloroplasts and thylakoids, I was never able to identify other organelles within the foraminifers, even in healthy individuals. Without this evidence for consistent, successful fixation, I could not draw any conclusions concerning changes associated with dormancy. Below I summarize my general fixation methods, as well as listing the variety of fixative formulas I tested. I then summarize concerns I had about these methods and suggestions for further development of methods that I was unable to address due to time and funding constraints.

General Methods

1. **Primary Fixation**
    a. See Primary Fixatives section for details
2. **Rinse**
    a. 3 changes 1 hour each rinse on rotor
    b. Following rinses tried:
        i. 0.1 M cacodylate buffered filtered seawater
        ii. Filtered seawater
3. **Decalcification A**
    a. Agar enrobe samples to maintain tissue orientation
        i. Make 1.5% agarose solution (15g SeaKem® Agar/L water)
        ii. Pour agarose into petri dish
        iii. Allow agarose to cool to 60°C or the point when the agar is almost solid
        iv. Rinse specimens with deionized (DI) water and then blot dry with paper towels
        v. Put specimens in nearly solid agar
        vi. Allow to cool
        vii. Use razor to cut out individual foraminifers in agar blocks
        viii. Trim as close to foraminifer as possible (~1mm on all sides if possible
        ix. Use sharp probe or hypodermic needle to push through agar until it touches shell, or scrape agar away until shell is exposed, on all sides of foraminifer
            1. Allows for penetration of decalcification solution to the shell itself
        x. Decalcify in 0.1M Ethylenediaminetetraacetic acid (EDTA) with pH of 6.8 for 3–5 days depending on size
        xi. Raise sample above container bottom so that decal solution penetrates all surfaces
        xii. Swirl decal solution in container several times a day to expose tissue to fresh solution
            1. May use mixing table on low for consistent swirl throughout the day
        xiii. Change decal solution once to twice daily
        xiv. Check each foraminifer daily - be careful to remove fragment from decal solution as soon as decalcified to avoid overexposure (leads to poor staining)
        xv. Rinse well with DI water (EDTA will precipitate in ethanol)
4. **Postfix**
    a. .Postfix in2% osmium tetroxide in DI water for up to 12 hours on rotor for better penetration; if overnight, put vial in frig. enclosed in a larger screw top bottle to catch osmium fumes.

b. .Followed by 2–3 15 min rinses in DI water on rotor
c. Rinse overnight in DI water on rotor

5. **Post-decalcification processing**
   a. Treat agar block as any block of tissue
6. **Dehydrate in Ethanol**
   a. 30%, 50%,70%; 90%, 90%, 100%, 100%
   b. 15 min each
7. **Embed**
   a. Used Embed 812

Primary Fixatives

1. Talge & Hallock, 1995
   a. 2.5% glutaraldehyde (GTA) and 2% formaldehyde (FA) in seawater for 12 hours
2. Talge & Hallock, 2003
   a. 2.5% glutaraldehyde and 2% paraformaldehyde in buffer of seawater
   b. 2.5% glutaraldehyde and 2% paraformaldehyde in buffer of 0.1M cacodylate (pH 7.4)
3. Bernhard et al., 2006
   a. 3% GTA in 0.1M sodium cacodylate buffer
4. Koho et al., 2018
   a. 2.5% GTA in seawater

Concerns and Suggestions

1. There were some general gaps in published methods; for instance, when not specified in the Primary Fixatives section, pH for fixative solutions was not provided.
2. *A. gibbosa* is large compared to many of the foraminiferal species examined using TEM in the literature; this makes methods and results difficult to easily compare.
3. Fixatives generally used at pH ~7.3, which is upper limit of pH for sodium cacodylate to effective buffer. This is slightly too acidic for foraminiferal shells, and led to decalcification occurring in fixative. As a result, samples cannot be stored indefinitely in fixative.
4. A primary problem throughout seemed to be efficacy of initial fixation. One possible solution could be vacuum fixation; however, this is difficult as the foraminiferal shell

seems to interfere with the bubbles that are generally used to indicate that appropriate pressure has been reached. Work would need to be done to refine this method; my early attempts seemed to cause membranes to lyse from pressure by the time the bubbles appeared.

5. Decalcification is necessary for thin sectioning of foraminifers, as calcium carbonate will chip the edges of diamond knives. However, care must be taken when decalcifying *Amphistegina gibbosa* specifically; due to the arrangement and thickness of chamber walls, decalficied cells will fall apart. Agar enrobement is necessary to support the cell and maintain orientation during decalcification. Placing foraminifers in agar seemed to have kept the cells together, but another possibility would be to try vacuum infiltration of agar.
6. Decalcification techniques used here may have led to issues with preservation, especially with membranes; I would suggest experimenting with less acidic solutions, or shorter time periods.
7. There may be concerns with pH and osmolarity throughout. Anywhere where DI water is used may benefit from replacement by seawater, buffer, or buffered seawater. In addition, I was not able to experiment with controlling osmolarity; appropriate techniques may lead to better fixation and preservation.
8. Uranyl acetate was used in some experiments as an en bloc stain, but lacking successful primary fixation, the benefits are unclear

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