MICROBIAL HOST-MUTUALIST COMMUNICATIONS IN MARINE

ORGANISMS: INSIGHTS FROM THE GIANT CLAM *HIPPOPUS HIPPOPUS*

By

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ABSTRACT OF THE THESIS

Microbial host-mutualist communications in marine organisms: insights from the giant

clam *Hippopus Hippopus*

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Mutualistic symbionts communicate with their hosts and can control host organ development. To determine if symbionts influence development in the giant clam *Hippopus hippopus*, I compared growth and cell proliferation in treatments of juveniles either inoculated or not inoculated (control) with *Symbiodinium sp.* Controls grew even without symbionts (1.03±0.41 µm/d, SE). Inoculated individuals grew significantly faster (2.91±0.37 µm/d) than control individuals ($P<0.001$). However, shell lengths did not significantly differ between treatments until day 22 post-fertilization, suggesting a delay in growth effects. Proliferating clam cells were randomly distributed ($P=0.99$) at day 13 but became non-randomly distributed ($P=0.002$) with increased proliferation within ~25 µm of a *Symbiodinium* at day 26. These results indicate that *H. hippopus* has a facultative period over which juveniles can still grow without *Symbiodinium*. I also reviewed current literature to determine what communication mechanisms may be inducing these effects in *H. Hippopus*. Host-mutualist interactions in giant clams and other marine species may involve a pathogen recognition mechanism such as MAMP/PRR pairing (including the prevalent lectin/glycan recognition), or the complement system.

DEDICATION

This work is dedicated to Dr. Diane K. Adams, who took on an uncertain graduate student and shaped a scientist through her unending enthusiasm for research and rigorous science. Throughout the past two years she provided guidance and assistance, giving new insights for topics beyond my experience, nudging me forward when I lacked direction, and allowing me freedom to independently pursue research once I gained my footing. She was truly an inspirational scientific role-model, and without her this thesis would not have been written.

husband, August, for his scientific and logical critiques as well as his patience and

undying encouragement.

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1. INTRODUCTION

In the ocean, hundreds of organisms host mutualistic microbes, either photosynthetic or chemosynthetic. Despite the abundance of published studies on mutualisms, the molecular mechanisms of communications between symbionts and hosts remain largely unknown. Researchers are only just beginning to understand the full extent of symbionts' effects on host development, including the benefits of probiotics (Balcázar et al. 2006). Marine organisms provide unique insights to these problems because of the accessibility of their symbionts for study. Their specialized symbioses also frequently involve only one or two symbiont species, making them easier to study than a diverse microbiota. Coral reefs are an abundant source of such mutualisms, where not only cnidarians but also bivalves, sponges, and nudibranchs harvest solar energy collected by symbionts. By studying marine organisms such as those in reefs, one can begin to tease apart some of the complexities of symbiont-host interactions in organisms from corals to humans (Kostic *et al.*, 2013).

1.1 Interactions between symbiont and host

Research on the microbiome in mammals has revealed a few intriguing insights, stirring interest in symbiont-host interactions. With recent developments in genomics and bioinformatics, researchers have been able to more fully characterize the human microbiome. We now know that the human microbiota is involved in development and maintenance of multiple tissues including bone (Sjögren *et al.*, 2012), immune tissue (Van De Pavert & Mebius, 2010), and the brain (Heijtz *et al.*, 2011). Because microbial

symbionts play such a necessary role in human development, altered microbiota are hypothesized to be involved in diseases from asthma to cardiovascular disease (O'Hara & Shanahan, 2006; Sommer & Bäckhed, 2013). The more scientists understand symbiont effects on host development in a variety of species, the better we can treat such diseases by learning how to encourage beneficial mutualisms or artificially provide the same benefits. Unfortunately, mammalian microbiomes are highly complex, involving multiple symbiont species within a single host. Such complexity increases the difficulty of studying individual aspects of symbiotic relationships. Therefore, researchers need model organisms that host easily isolated symbiotic relationships with an individual mutualist species supporting one symbiotic function, offering a level of experimental control not possible in humans (Kostic *et al.*, 2013).

Several marine organisms meet the requirements for a model organism, having one or two easily isolated symbionts and partnerships relevant to scientific questions. Studies on the Hawaiian bobtail squid *Euprymna scolopes,* which possesses a highly studied extracellular bacterial symbiont in a specialized light organ (Nyholm & McFall-Ngai, 2004), and *Osedax* boneworms, hosts to bone-digesting bacteria (Nussbaumer *et al.*, 2006), have shown that symbiotic relationships can be horizontally transmitted and established. Corals and other cnidarians are of great interest, since understanding their endocellular symbiotic relationship with photosynthetic dinoflagellates may help develop methods to protect coral reefs from bleaching (Douglas, 2003). Several bivalve molluscs can associate with either photosynthetic or chemosynthetic symbionts and house their symbionts extracellularly in the gills (Southward, 1986) or, in the case of giant clams, a mantle-pervading tubular system (Norton *et al.*, 1992, see Fig. 1). Each marine model

organism can be studied for unique insights into different aspects of symbiont-host

relationships in general (Kostic *et al.*, 2013).

1.2 Symbiont-host interactions in growth and development

Within the context of host-symbiont interactions, one mystery is how symbionts

induce changes in growth and development of their hosts. We know that symbionts

modulate aspects of host development. Mouse brains do not develop normally without

gut microbiota and the axenic mice are unusually anxious, for instance (Heijtz *et al.*,

2011). However, the way which microbes induce changes in developing host organs is

not well understood. Symbionts might allow the enhanced absorption of certain nutrients

or a larger amount of food. However, developmental effects, rather than increased growth

rates alone, are observed in multiple hosts (Sommer & Bäckhed, 2013). These

developmental effects imply that symbionts interact with the host to induce

developmental changes.

Species-specific symbiont selection found in multiple hosts also implies a host-

symbiont interaction in development. In the giant clam *Hippopus hippopus,* juvenile

clams ingest multiple species of algae (Fitt & Trench, 1981), but only *Symbiodinium* spp.

can avoid digestion and travel into the host tissue to establish a symbiotic relationship

(Hirose *et al.*, 2006). Juvenile clam survival increases as much as three times when raised

with an isolated *Symbiodinium* strain from a host of the same species in contrast to strains

from other species, or even from hosts within the same genus (Fitt & Trench, 1981). This

host specificity and the algae's resistance to digestion suggest a communication between

host and symbiont. Favoring particular symbiotic microbes over other microbes by host

species has also been observed in legumes (Cooper, 2007), deep-sea mussels, (Détrée *et al.*, 2017), and bobtail squid (McAnulty & Nyholm, 2016), further hinting at a communication between symbionts and hosts to aid recognition.

Giant clam tubular systems provide additional evidence of host-symbiont communication. The tubular system is a specialized structure in the mantle, which giant clams use to house their symbionts (Norton *et al.*, 1992). It develops around the time symbiosis is established at 2-4 weeks (Hirose *et al.*, 2006), suggesting symbionts may be involved in tubular system initiation. Giant clams can bleach as corals do, expelling or losing their symbionts (Norton *et al.*, 1995). After bleaching, their tubular systems atrophy, suggesting that *Symbiodinium* may be involved in maintaining the tubular system via an extracellular signal (Norton *et al.*, 1995).

1.3 Summary

Symbionts are essential to the development of host organisms from tubeworms to mice, but researchers are still teasing apart signaling mechanisms. Current research suggests that symbionts can not only increase host growth rate but also induce development of certain organs. By understanding mechanisms by which symbionts and hosts interact, we can learn more about symbionts' developmental effects on hosts and apply this knowledge in additional contexts, such as the human microbiome. This work will explore marine host-symbiont interactions, using the giant clam-*Symbiodinium* symbiosis as a case study. Chapter 2 describes a study performed to determine whether symbionts influence growth and cell proliferation in giant clams. Chapter 3 describes

known and potential pathways for host-symbiont communication, and the final chapter

provides a concluding discussion.

[1]2. FACULTATIVE ACQUISITION AND *SYMBIODINIUM*-LOCALIZED CELL PROLIFERATION IN THE GIANT CLAM *HIPPOPUS HIPPOPUS*

2.1 Abstract

Giant clams (subfamily Tridacnidae) house their obligate symbionts, *Symbiodinium* sp, in a specialized tubular system. Rapid uptake of *Symbiodinium* has been shown to be necessary for early clam survival, suggesting that the symbionts play an essential role in host growth and possibly development. To determine whether symbionts influence development in the giant clam *Hippopus hippopus*, I compared growth patterns and cell proliferation in two treatments of juveniles inoculated or not inoculated (control) with *Symbiodinium sp.* Symbiont uptake occurred continuously from days 8 to 26, with ~5% d^{-1} of individuals colonized on average. The control culture grew even without symbionts (1.03 ± 0.41 µm d^{-1}, standard error). Inoculated individuals grew significantly faster (2.91 ± 0.37 µm d^{-1}) than control individuals ($P < 0.001$). However, daily shell length measurements did not significantly differ between the inoculated and control cultures until day 22, suggesting a delay in growth effects. Consistent with this, at day 13, proliferating clam cells were not correlated with symbiont abundance, ($P = 0.13$); while at day 26, proliferating clam cells were correlated with symbiont abundance ($P < 0.01$). In inoculated individuals, the proliferating cell pattern also changed from being randomly distributed ($P = 0.99$) at day 13 to non-randomly distributed ($P = 0.002$) with increased likelihood of proliferation within ~25 µm of a symbiont at day 26. My results indicate that juvenile *H. hippopus* has a longer, facultative *Symbiodinium* acquisition period than

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[1] A version of this chapter is currently in review with *The Biological Bulletin*.

previously recorded, after which proliferation and development is enhanced but during which growth is unaffected by *Symbiodinium*.

2.2 Introduction

Giant clams maintain reef ecology by providing flesh and discharges that are food for predators and scavengers, shells that form shelters and enrich the habitat, and water filtration (Heslinga and Fitt, 1987; Neo *et al.*, 2015). They are an important economic product for people – providing meat and shells and resulting in an ongoing poaching problem (Gomez and Mingoa-Licuanan, 2006; Guest *et al.*, 2008; Neo *et al.*, 2015; Tisdell *et al.*, 1993). In addition to providing important economic benefits, giant clams (subfamily Tridacnidae) make a useful model for studying host-symbiont interactions. While intracellular *Symbiodinium* spp. in corals are difficult to observe within the cells, Giant clams' intercellular symbionts, *Symbiodinium* sp., are easily observed and thus readily studied.

Inoculation of giant clams with *Symbiodinium* is believed to be necessary within the first week to ten days of development for proper growth, but the idea that symbionts are obligate in juvenile giant clams warrants further investigation. A non-obligate relationship within the first few weeks would benefit the clam host by enabling it to settle and begin feeding without initially relying on symbionts. Previous studies indicate that *Symbiodinium* induce a higher growth rate in giant clams and increase juvenile survival (Fitt and Trench, 1981; Fitt *et al.*, 1986; Mies *et al.*, 2012). However, these experiments measure growth before and after symbionts are provided rather than comparing larvae with and without symbionts. It is often recommended and standard practice for

aquaculturists to seed giant clam juveniles with *Symbiodinium* within the first week to prevent mass mortality (Fatherree, 2006). Other observational experiments indicate that giant clam juveniles will not metamorphose without *Symbiodinium* (Fitt *et al.*, 1984; Gwyther and Munro, 1981), an idea still prevalent in aquaculture in the Republic of Palau (personal communication). In the wild, an individual might not encounter the correct symbiont in time for normal metamorphosis, which would make this requirement detrimental to survival. However, to the best of My knowledge, studies examining giant clams for more than 5 days post-fertilization have not included a control group wherein clams were fed algae but not inoculated with *Symbiodinium*. Fitt and Trench (1981) performed experiments with unfed giant clam larvae lacking *Symbiodinium,* but these larvae did not live beyond day 10. The lack of a fed control leaves questions about whether the giant clam-*Symbiodinium* relationship is indeed obligate.

The variability in timing of symbiont acquisition within a cohort is likewise unknown. Mies *et al.* (2012) noted an immediate increase in growth rate in *Tridacna crocea* juveniles after adding *Symbiodinium*. On the other hand, Fitt and Trench (1981) argued that growth rate increased significantly only after *Symbiodinium* had fully traversed the mantle tissue. Some have suggested that symbiosis does not even begin during larval development (Mies *et al.*, 2017). Multiple species of giant clams (*T. squamosa, T. gigas,* and *H. hippopus*) begin to acquire symbionts within 7-10 days post-fertilization when provided with *Symbiodinium* on hatching (Fitt and Trench, 1981; Fitt *et al.*, 1984; Norton *et al.*, 1992), but the period over which a cohort of clams can acquire symbionts has not been determined.

If symbiosis in giant clams is obligate in early development, the obligation may be due to either nutritional requirements or developmental benefits. Giant clams harvest photosynthetic products from their symbionts (Goreau *et al.*, 1973; Muscatine, 1967; Streamer *et al.*, 1988; Trench, 1979; Trench *et al.*, 1981). They also digest some *Symbiodinium* (Yonge, 1936), usually those that are already dead or weakened (Fankboner, 1971; Trench *et al.*, 1981). Even so, symbionts supply less than half of nitrogen requirements (Hawkins and Klumpp, 1995) and 35-66% of total carbon requirements (Klumpp *et al.*, 1992) in adult clams. Filter feeding supplies the remaining nutritional needs in adults. Juveniles also filter feed (Fitt and Trench, 1981), but the relative contributions of filter feeding and symbiosis to nutritional needs of larvae and juveniles is unknown. Further experimentation is needed to evaluate the symbiont contribution to overall juvenile clam nutritional requirements.

Some evidence indicates that *Symbiodinium* may induce developmental changes in giant clams, including affecting giant clam cell proliferation in the tubular system (Hirose et al. 2006). In other organisms, we now know that microbiota have functions involving mediation of host cell proliferation (Buchon *et al.*, 2009; Renz *et al.*, 2012; Sommer and Bäckhed, 2013). Gut microbiota moderate the proliferation of the mouse lymphoid system and T-cell generation (Hooper *et al.*, 2012). They also directly impact proliferation of the host intestinal epithelium (Smith *et al.*, 2007), brain (Heijtz *et al.*, 2011) and bone (Sjogren *et al.*, 2012). Symbiotic bacteria reshape the developing light organ in the bobtail squid *Euprymna scolopes* by causing death of some host cells and proliferation of others (Montgomery and McFall-Ngai, 1994). The giant clam tubular system is an organ which functions specifically in symbiosis, similarly to the light organ

of *E. scolopes* (Norton *et al.*, 1992). Thus, *Symbiodinium* may influence tubular system development and maintain it throughout the clam's life, as evidenced by loss of tubules in bleached clams (Norton et al. 1992). Although juvenile giant clams may not need symbionts for nutrition and growth, the symbiosis may be obligate for developmental processes when development of the symbiosis-related tubular system is initiated. In each case, the specific mechanisms by which microbes induce changes in host development remain largely unknown.

In this study, I investigate the influence of *Symbiodinium* on early growth rates and cell proliferation in juvenile *Hippopus hippopus* (Linnaeus, 1758) giant clams. For this purpose, I controlled for the presence of *Symbiodinium* by using fed clams with and without symbionts provided. I determined growth and cell proliferation rates, both in the presence and absence of *Symbiodinium,* to investigate when symbionts begin to affect the host. I also assessed the proximity of proliferating cells to symbionts in order to determine if *Symbiodinium* encouraged localized cell proliferation. I discuss whether and how symbiosis is facultative in the first four weeks of giant clam development, and if symbionts affect cell proliferation.

2.3 Materials and Methods

2.3.1 Culture and experimental setup

Symbiotic and asymbiotic development was compared using cultured clams while controlling for inoculation with *Symbiodinium*. I obtained approximately 2.1 million 6-day post-fertilization veliger larvae of *H. hippopus* from the Palau Bureau of Marine Resources. I set up six plastic tubs filled with 1.5 L aerated seawater each and an initial

larval density of ~1.3 larvae mL^{-1} at the Palau International Coral Reef Center; excess

larvae were maintained in a backup stock tank. I collected *Symbiodinium* sp. from a

sacrificed adult clam. On day 6 post-fertilization, the first day of the experiment, I added

the *Symbiodinium* treated with 10 ppm streptomycin sulfate as an antibiotic to three of the

cultures with unfiltered seawater, henceforth the inoculated treatment. The three

remaining cultures without *Symbiodinium* functioned as the control treatment. Each tub

was fed approximately 500 cells mL^{-1}d^{-1} Instant Algae (*Isochrysis* 1800, Reed

Mariculture Inc.) (Fitt *et al.*, 1986), with 50% water changes every 5 days. I performed an

additional 20% water change on day 7 to reduce excessive surface algal growth. I

maintained water conditions at salinity 30.39 ± 0.15 ppt (standard error, SE), pH 8.11 ±

0.01 (SE), and temperature 28.60 ± 0.02 ℃ (SE) to approximate local conditions. HOBO

Pendant data loggers (Onset Computer Corporation) recorded temperature and luminosity

in each tub every 15 minutes. Temperature, pH, and conductivity were measured daily at

09:00 Palau time (PWT) using an Accumet AP85 handheld meter (calibrated once per

week according to manufacturer directions). Cultures were held near a northwest-facing

window in indirect sunlight.

2.3.2 Growth

To determine growth rates, I photographed a minimum of six clams (range 6-33)

drawn daily in a random 10 mL seawater sample from each treatment using a Zeiss

Axiocam 105 color camera at 50x optical magnification attached to a Zeiss Stemi 508

microscope. I measured the shell length of each clam photographed across the longest

axis (from anterior to posterior) using Zen 2 Blue edition software.

To account for repeated measures in the shell length measurements, I ran MANOVA tests with shell length as the dependent variable and with time and treatment as independent variables. I tested whether the control and inoculated treatments differed in shell length across all 20 days of the experiment using MANOVA. I then ran univariate ANOVA tests as post-hoc pairwise comparisons on the shell length measurements to test where the divergence occurred, with shell length as the dependent and treatment (inoculated or control) as the independent variable. I ran a MANOVA restricted to the first 4 days of the experiment (6-9 days post fertilization) to test for initial or immediate length differences between clams in the control and inoculated treatments. This period from days 6-9 was chosen to exclude influence of symbiosis, since ~70% of clams remained uncolonized at day 9 and only ~10% of clams had reached late stage symbiosis.

2.3.3 Establishment of symbiosis

For each clam imaged for growth (above), I recorded the stage of symbiosis. I defined three stages of *Symbiodinium* colonization, modified from Fitt and Trench (1981) and Hirose et al (2006). Fitt and Trench (1981) marked symbiosis establishment by the propagation of symbionts outside the stomach and up the side of the clam. Hirose *et al.* (2006) defined establishment as propagation of symbionts across the ventral mantle edge. For the purposes of this research I defined each of these points as separate stages in the progression of symbiosis following Fitt and Trench (1981). I defined the stages as follows: "Uncolonized" clams had no *Symbiodinium* visible or only had algae in the stomach region. "Early" stage clams had *Symbiodinium* extending up the side but not

across the ventral region. Finally, "late" stage clams had *Symbiodinium* extending across

the ventral mantle region (illustrated in Figure 2).

To predict the point when on at least 50% of clams are colonized, I performed a

logistic regression where the stage of symbiont infection was a categorical response

variable and time was a continuous predictor variable in R (v. 3.4.0 (R Core Team,

2014)). To compare symbiont acquisition rates, I performed t-tests between linear

regressions to see if acquisition rates differed significantly between the treatment and the

control.

2.3.4 Cell proliferation

I labelled individuals with EdU cell proliferation stain on days 13 and 26 to assess

locations of cell division within the clams at the midpoint and end of the experiment

(Salic and Mitchison, 2007). I selected day 13 because 50% the inoculated clam cohort

was uncolonized at that time (see prior section for colonization timing analysis). Day 26

represented an endpoint where 100% of inoculated clams were colonized. I included

EdU-negative controls and sampled each treatment in triplicate. For each sample,

approximately 20 larvae were collected, rinsed over a 60-µm filter with 1-µm filtered

seawater, and placed in 1 mL filtered seawater with 10 µM EdU (Life Technologies). The

larvae were incubated at 28-30 °C for 24 h under the same conditions as the main

cultures. Samples were fixed in 5-10 mL 4% formaldehyde in PBS for 12 h, washed 3x

with PBS, and stored temporarily at 4 °C in 1% formaldehyde in PBS. I detected EdU in

labeled samples with the Click-iT Plus Alexa Fluor 488 picolyl azide toolkit (Life

Technologies) following manufacturer directions. I counterstained for nuclei with 300

nM DAPI Nucleic Acid Stain (Thermo Fisher Scientific) in PBS for 5 min per manufacturer directions. Stained samples were stored in 0.02% sodium azide at 4 °C.

We imaged EdU-labelled clams and controls at 40x magnification using a Zeiss LSM 710 confocal microscope to quantify EdU labeling. I used a 405-nm laser to illuminate DAPI, 488-nm for Alexa-flour (EdU), and 594-nm for the natural fluorescence of *Symbiodinium*. Three dimensional images were rendered using Imaris 8.1 software from Bitplane. The numbers of DAPI^{+} cells, EdU^{+} cells, and *Symbiodinium* were measured in Imaris. The number of EdU^{+} cells was normalized to the number of DAPI^{+} cells for each individual clam to account for size differences. I measured the distance from the center of the nearest DAPI[+] clam cell to the center of each *Symbiodinium* and the distance from the center of the nearest *Symbiodinium* to the center of each EdU[+] clam cell.

We ran a two-sample Kolmogorov-Smirnov (KS) test to determine if the distances between EdU^{+} cells and *Symbiodinium* were randomly distributed (Fasano and Francheschini, 1987). I compared this to 5000 randomly-generated distributions to determine significance. I determined correlation between the number of *Symbiodinium* and the number of EdU^{+} cells on days 13 and 26 using a student's t-test on the Pearson correlation coefficient. I examined whether individuals on day 26 had significantly more EdU^{+} cells than those on day 13 using a student's t-test.

2.4 Results

2.4.1 Growth

No initial difference in shell length existed between the inoculated and control treatments, but the treatments diverged over the 20 days of the experiment (Fig. 3). Shell length significantly differed both across days (Pillai's Trace = 0.383, $F = 16.1$, $df = 491$, $P < 0.001$), indicating growth of the juvenile clams over time; and between treatments (Pillai's Trace = 0.023, $F = 11.4$, $df = 491$, $P < 0.001$), indicating that the inoculated treatment attained significantly larger shells than the control over 20 days. The MANOVA test restricted to the first 4 days showed a significant effect for shell lengths across days, consistent with the growth of juvenile clams over time (Pillai's Trace = 0.095, $F = 3.23$, $df = 92$, $P = 0.0260$). However, it showed no significant multivariate effect for shell lengths between treatments (Pillai's Trace = 0.019, $F = 1.79$, $df = 92$, $P = 0.184$).

Post-hoc tests estimating the time at which shell lengths began to differ showed a significant divergence in shell length between treatments at day 22 post-fertilization. Post-hoc pairwise comparisons showed significant differences in shell length between treatments on days 8 ($P = 0.0311$), 11 ($P = 0.0122$), 22 ($P = 0.0123$), 23 ($P = 0.0028$), 25 ($P < 0.001$), and 26 ($P = 0.0058$). The tests on days 13 (non-significant result) and 26 (significant result) are particularly relevant since they correspond to ~ 30% and 100% colonization and are supported by similar results in the cell-proliferation data discussed below. Days 8 and 11 appear to be outliers since days 9-10 and 12-21 showed no significant differences.

The inoculated treatment showed a greater shell growth rate than the control treatment (Fig. 3). Across the experiment, the control treatment's shells grew at a rate of 1.03 ± 0.41 μm d^{-1} (95% CI), whereas the inoculated treatment's shells grew at a rate of

2.91 ± 0.37 µm d^{-1} (95% CI). A two-tailed t-test confirmed that the overall shell growth

rate differed significantly between treatments ($P < 0.01$), with the inoculated treatment

showing a higher rate of shell growth. Individuals in this treatment measured on average

21.0 µm longer than those in the control treatment on days 22-26.

2.4.2 Symbiont acquisition

Symbiont colonization of the host occurred throughout the 20 day experiment and

was completed by the end of the experiment (Fig. 4). *Symbiodinium* colonization started

on day 8, two days after symbiont introduction. By day 26, 100% of the observed

juveniles were colonized. A logistic regression fit to the observations of colonization

stage shows that colonization and *Symbiodinium* establishment occurred gradually and

fairly consistently from day 8 to day 26. The point at which 50% of individuals are

colonized, as predicted by the logistic model, is at day 17.4 (Fig. 4). At day 13, the cohort

is predicted to be ~30% colonized, whereas at day 26, the cohort is 84% colonized. Thus,

days 13 and 26 are subsequently used as reference points for comparison prior to and

after colonization.

2.4.3 Cell proliferation

Confocal microscopic observations revealed that the number of EdU^{+} clam cells

in an individual over a 24-hour exposure (34.7 ± 34.0) was correlated with the number of

Symbiodinium colonizing an individual (44.1 ± 54.2) on day 26 (n=8, P < 0.01, Fig. 5B).

No correlation existed on day 13 (n=25, $P = 0.13$, 7.8 ± 3.7 EdU^{+} clam cells, 6.2 ± 4.6

Symbiodinium Fig. 5A), indicating that the effect of symbionts on host cell proliferation

occurs later in development or the symbiont colonization process. The average number of

DAPI$^+$ cells per clam, an estimate of the number of cell nuclei, was 1031 ± 81 (95% CI).

The average number of *Symbiodinium* within each clam increased from 6.2 ± 4.6 at day

13 to 44.1 ± 54 (95% CI) at day 26 (Fig. 6). Juveniles at 13 days old had few EdU$^+$ cells

$(7.8 \pm 3.7$, 95% CI) even at a late stage of *Symbiodinium* colonization (Fig. 6C),

indicating a low level of cell proliferation. By day 26, juveniles had significantly more (P

$= 0.04$) EdU$^+$ cells (34.7 ± 34.0 cells, 95% CI), especially at a late stage of colonization

(Fig 6F). Interestingly, on observation of cell division in *Symbiodinium, Symbiodinium*

cells showed rapid cell division as evidenced by EdU staining, with 27.9 ± 17.9

Symbiodinium dividing on day 13, and 70.0 ± 17.9 on day 26 (95% CI).

Measurements of distances between *Symbiodinium* and EdU$^+$ clam cells or DAPI$^+$

clam cells (Fig. 7) showed that cell proliferation was more closely associated with

symbionts at day 26. The distance between the centers of DAPI$^+$ cells and the centers of

Symbiodinium remained unchanged between days 13 and 26, and was on average $8.20 \pm$

0.96 μm (SE). The distances between the centers of EdU$^+$ cells and the centers of

Symbiodinium formed a clear peak on day 26 that was closer to the *Symbiodinum* on day

26 than on day 13. A modified Kolmogorov–Smirnov (KS) test examined whether the

distribution of distance measurements between EdU$^+$ cells and *Symbiodinium* was

different from random distributions. The closest EdU$^+$ cell neighbors were randomly

distributed on day 13 ($P = 0.99$); and 32.0% of *Symbiodinium* had an EdU$^+$ neighbor

within 24 μm. However, on day 26, the distribution was non-random ($P = 0.002$) with a

peak at 20 μm (~2.2-2.8 cell lengths). 45.6 % of *Symbiodinium* had an EdU$^+$ neighbor

within 24 μm. At both time points, 95.6% of *Symbiodinium* had a DAPI[+] neighbor within 24 μm (~ 2.6-3.3 cell lengths).

2.5 Discussion

After an approximately three-week facultative colonization period, *Symbiodinium* began to increase the early juvenile growth of *H. hippopus* by supporting faster growth and by increasing host cell proliferation in the immediate vicinity of symbionts. Clams with *Symbiodinium* had higher cell proliferation rates and grew faster than those without symbionts, but this occurred later than expected based on previous studies showing effects of symbionts immediately (Mies *et al.*, 2012), or a requirement for *Symbiodinium* by day 10 (Fitt and Trench, 1981). Increased growth and cell proliferation rates were not detected until clams were three to four weeks old even though by this time most clams had acquired symbionts. If symbionts are needed for host development, the relationship appears to become obligate between days 13 and 26 in *H. hippopus*. This finding suggests a significantly longer facultative period exists, lasting at least until day 13, for the *H. hippopus* giant clam-*Symbiodinium* symbiosis than previously indicated for giant clam species (Fatherree, 2006; Fitt and Trench, 1981; Fitt *et al.*, 1984; Gwyther and Munro, 1981). My study was limited to this single species of giant clam based on availability. However, *H hippopus* is one of the most commonly cultured and studied giant clam species and I consider it representative of giant clams in general.

This result adds new understanding to previous studies on giant clam growth and symbiosis. In agreement with previous research (Fitt and Trench, 1981; Fitt *et al.*, 1986) showing that *Symbiodinium* containing clams grow faster, especially when symbionts

have travelled fully across the mantle region, these results show improved growth once

H. hippopus is fully colonized by the symbiont. In contrast, these results show new

evidence that increased growth due to *Symbiodinium* only occurs at late stage symbiosis,

starting around day 22 for *H. hippopus,* rather than immediately when symbionts are

taken up. Also, my results suggest that when fed the non-symbiotic algae *Isochrysis,*

juvenile clams continue to survive and grow even without symbionts. Uncolonized clams

do not grow as quickly as colonized clams after day 22 (Fig. 3). This result counters some

studies which indicate that some giant clam species cannot survive more than 10 days or

even metamorphose without symbionts present (Fitt and Trench, 1981; Fitt *et al.*, 1984;

Gwyther and Munro, 1981). Some studies indicate that the giant clam *T. squamosa* does

not grow without symbionts (Fitt and Trench, 1981); however, those clams were not

provided microalgae on which to feed as an alternative food source. The knowledge that

giant clams can grow for a time without symbionts so long as they have microalgae to

feed on may prove useful for planning the introduction of symbiotic algae to a culture. A

second dose of *Symbiodinium* may improve symbiosis establishment in giant clams

which do not immediately acquire symbionts, and provision of microalgae may improve

survival of a culture.

It is possible that microalgal density was too low at 500 cells/mL to sustain

asymbiotic giant clam growth past day 26 in this experiment, although that concentration

is recommended in giant clam aquaculture (Fitt *et al.*, 1986). The slowdown in growth in

Figure 3 might indicate a growth plateau at that point, but whether this is due to limited

food availability or lack of symbionts is not certain. There may be a threshold density of

microalgal food required for giant clam juvenile growth without symbionts. In an

oligotrophic system such as Palau's coral reefs, a low concentration of algae may be realistic, but further investigation is required. It is also possible that time of exposure and dose of symbionts affects the results seen in this work. Further experimentation is required to account for such possibilities.

In addition to gaining new insights on growth and the facultative nature of symbiosis in giant clams, I have shown that cell proliferation rates increase in proximity to *Symbiodinium*. More clam cells divide within a few cell lengths of a symbiont than in other areas of the clam at day 26, compared to a random distribution at day 13. The non-random clustering of dividing cells around symbionts may indicate a direct communication between *Symbiodinium* and the host to stimulate proliferation at day 26. Hirose *et al.* (2006) saw evidence of tube structures in *T. crocea, T. derasa,* and *T. squamosa* after 2 weeks of age, after early stage symbiosis had occurred. Epithelial tubule tissue was clearly visible at day 36 post-fertilization. Likewise herein, cell proliferation was observed to increase at day 26 after *Symbiodinium* migrated into the mantle. My findings, combined with those of Hirose *et al.* (2006) that show tubules beginning to form around symbionts as young as day 14, suggest that the clam tubular system develops around the symbionts and that *Symbiodinium* presence may induce tubular system growth. Further evidence is provided by the ascension of symbionts into the mantle tissue and the rapid division rates of these symbionts, indicating that the *Symbiodinium* are actively proliferating to colonize available space rather than being digested by the host. Rather than a symbiosis not being set up at all during larval development (Mies *et al.*, 2017), I conclude that symbiosis is established later in development than previously assumed.

Questions remain about the mechanism of communication between *Symbiodinium* and the host to stimulate proliferation. It is possible that nutritional products induce host growth, or that developmental cues orchestrate the growth of symbiosis-related structures apart from initiation by the symbionts. One potential avenue of exploration is to tease apart the developmental versus nutritional effects of *Symbiodinium* in giant clams. Biochemical or genetic pathways and mechanisms may help explain how symbionts influence development apart from providing nutritional products, but lack of a published genome for any species of giant clam has hampered such mechanistic insight of symbiont-host interaction. However, some pathways have been identified in other symbioses which may also be involved in the giant clam-*Symbiodinium* symbiosis. For instance, the complement pathway has been linked to initiation and maintenance of symbionts in cnidarians (Poole *et al.*, 2016).

Our results support the conclusion that *H. hippopus* juveniles grow faster with *Symbiodinium* than without after a three-week period of facultative colonization. Increased cell proliferation localized in proximity to symbionts in later stages of colonization also implies that *Symbiodinium* may induce development of the tubular system, known to develop around week 2 post-fertilization (Hirose et al. 2006). More broadly, understanding how an intercellular symbiont, such as that in the giant clam-*Symbiodinium* relationship, communicates with and influences the host could provide new insights into other such symbioses, from the human gut microbiome to shipworms.

[2]3. MECHANISMS OF HOST-SYMBIONT COMMUNICATION IN MARINE MUTUALISMS

3.1 Abstract

Hosts and symbionts in marine mutualisms communicate to facilitate uptake and recognition of symbionts, as well as direct changes necessary for the symbiosis to develop. Known mechanisms for communication are the complement system, Microbe-Associated Molecular Patterns (MAMPs) with Pattern Recognition Receptors (PRRs), and (minorly) cell-size and ApRab protein recognition. The complement system is a component of innate immunity known to be involved in recognizing and attacking pathogens. Complement genes for Factor B and Mannose-binding lectin-Associated Serine Protease (MASP), among others associated with the lectin and alternative pathways, are differentially expressed at onset and during maintenance of symbiosis. Also, invertebrates use MAMPS to recognize microbes by means of PRRs on microbial cell surfaces. Peptidoglycan Recognition Proteins (PGRPs), C-type lectin-like receptors, *Sinularia lochmodes* Lectin 2 (SLL-2), *Ctenactis echinata* Lectin (CecL), galectin, and other lectins are all PRRs associated with mutualisms. These PRRs recognize cell-surface glycans of the common marine symbiont, *Symbiodinium* spp. Finally, ApRab proteins and cell size are also used by some marine organisms to identify and acquire symbionts. In this literature review, I found that each marine mutualism involves unique recognition systems and developmental effectors, but generally symbiont-host communication mechanisms are closely bound to the pathogen-recognition framework.

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[2] This chapter is intended for submission to *Marine Biology.*

3.2 Introduction

        Mutualistic symbionts directly affect development and physiology of marine organisms, and enormous progress has been made in understanding how symbionts and hosts communicate to initiate these changes (Sommer and Bäckhed 2013). A few types of communication are needed to establish and maintain symbiosis. First, either a host must differentiate symbionts from pathogens, or symbionts must evade host immune responses and/or digestion to initiate symbiosis. In order to encourage a suitable environment for symbiosis, symbionts may induce changes in the host (Montgomery and McFall-Ngai 1994). For instance, in the Hawaiian bobtail squid, *Euprymna scolopes,* the bacterial symbiont *Vibrio fischeri* initiates development of symbiotic light organs (Montgomery and McFall-Ngai 1994). The development of symbiosis-specific organs such as the light organ in *E. scolopes* and the tubular system in giant clams (Norton *et al.* 1992), suggests that symbionts actively communicate with the host for recognition and to trigger developmental changes. When it comes to communication and developmental effects in a mutualistic relationship, distinguishing the roles of symbiont and host often proves challenging. This is especially true in obligate relationships where one or both symbiotic partners cannot be raised independently, but experimental design can be made easier in species for which gnotobiotic (colonized only by known organisms) or asymbiotic individuals can be raised (Ezenwa *et al.* 2012). Integrating accumulated knowledge of symbiotic roles across multiple mechanisms and taxa may help elucidate the workings of varied and complex symbioses, from deep-sea chemosynthetic to coral reef photosynthetic symbioses.

Microbial symbionts are known to induce changes in their hosts. For instance, in insect-parasitizing nematodes, the bacteria *Photorhabdus luminescens* induces nematode juveniles to grow and develop into adults (Strauch and Ehlers 1998). Also, loss of bryostatin-producing symbionts in the marine bryozoan *Bugula neritina* results in fewer reproductive ovicells and may reduce host reproductive ability (Mathew *et al.* 2016). *Symbiodinium* may also affect host gene expression throughout host lifespan, as evidenced by greater expression of the glycosylated membrane protein Sym32 when *Symbiodinium* (as opposed to green algae) are present in the anemone *Anthopleura elegantissima* (Reynolds *et al.* 2000). The coral *Acropora digitifera* also shows differential expression in three percent of its transcriptome at four hours after *Symbiodinium* exposure (Mohamed *et al.* 2016).

Symbionts induce developmental changes and differential gene expression in host organisms, although researchers have not fully explained how these effects are accomplished. However, a few mechanisms have come to light as possible methods of host-symbiont communication. Weis (2008), among others, suggests that host-symbiont recognition may involve host innate immunity modulated through the release of chemical species such as nitric oxide (NO) and reactive oxygen species (ROS). Kvennefors and colleagues (2008) note that although only vertebrates possess antigen-specific adaptive immunity, both vertebrates and invertebrates possess innate immunity, a generic pathogen defense system. They explain that innate immunity involves an ability to recognize and bind to foreign microorganisms (often pathogens). They furthermore describe that recognition frequently activates molecular signals leading to agglutination

and phagocytosis of foreign bodies – a step also necessary in acquiring endocellular symbionts.

Two main pathways have been identified that use a pathogen-recognition system to identify mutualistic microbes: the complement system, which can recognize and destroy pathogens or allow symbionts past host immunity; and recognition of Microbe-associated Molecular Patterns (MAMPs) on pathogens and symbionts by host Pattern Recognition Receptors (PRRs) (Castillo *et al.* 2009; Kilpatrick 2002; Kimura *et al.* 2009; McAnulty and Nyholm 2016; Poole *et al.* 2016). Other mechanisms involve cell size and ApRab proteins (see Table 1 for summary) (Biquand *et al.* 2017; Fransolet *et al.* 2012). One or more of these mechanisms is probably involved in giant clam symbiosis. The developmental interactions of host and symbiont in *Hippopus hippopus* indicate a communication between the two partners.

In summary, host-symbiont communication is necessary for organisms to identify appropriate symbiotic partners, prevent destruction of symbionts by a host, and induce changes in each partner required for mutualisms. Currently, known communication mechanisms mirror pathogen-recognition systems. Rather than destroying symbiotic microbes, these mechanisms allow or encourage symbiosis establishment and maintenance. In giant clams, the complement system may be involved in initiating development of the tubular system around *Symbiodinium* between 2-5 weeks post-fertilization (see Chapter 2). This review of current literature will explore known mechanisms and their involvement in symbioses, focusing on the complement system and on MAMP/PRR interactions but also mentioning a few other possible pathways.

3.3 Known mechanisms

3.3.1 The complement system

The complement system is a molecular cascade essential for innate immunity in organisms ranging from corals to humans, and for adaptive immunity exclusively in vertebrates (Ricklin *et al.* 2010). Its main function is to attack and eliminate microbes. However, it also "complements" other forms of immunity and inflammation and is involved in a diverse range of physiological processes. Depending on the organism, one or more of three complement pathways may be present: classical, alternative, or lectin (see Fig. 2 in Nonaka 2011). While all three pathways are found in vertebrates, only alternative and lectin pathway proteins are found in invertebrates.

Invertebrate complement genes are evidently constitutive in marine mutualisms as part of innate immunity, but are also involved in symbiotic recognition. Complement gene expression has been associated with symbiotic processes in *E. scolopes* and in the sea anemones *Nematostella vectensis* and *Aiptasia pallida,* among others (Castillo *et al.* 2009; Kimura *et al.* 2009; Poole *et al.* 2016). In *E. scolopes,* the complement protein C3 is constitutively expressed from hatching to adulthood, as should be expected of an immune pathway (Castillo *et al.* 2009). However, Ap_Bf-1 (*Aiptasia pallida* Factor B1) and Ap_MASP (*A. pallida* Mannose Binding Lectin-Associated Serine Proteases), both involved in initial bacterial recognition steps, are upregulated at onset of symbiosis in *A. pallida,* indicating involvement in communication with or recognition of symbionts (Poole *et al.* 2016).

At a basic level, the classical complement pathway uses a cascade of molecules to destroy pathogens (Müller-Eberhard 1985). Upon recognition of a bacterial cell,

complement Factor C1q initiates the classical complement pathway by binding to and activating complement factors C1r and C1s (Gaboriaud *et al.* 2004; Ricklin *et al.* 2010; Sunyer *et al.* 1998). Next, C1s acts as a C4 convertase leading to opsonization (identification of a particle as a target for phagocyte destruction) and generation of C3 convertase (or C4b2b) (Ricklin *et al.* 2010). Then C3 convertase cleaves C3 and amplifies the pathway by activating downstream receptors. The downstream receptors C5, C6, C7, C8, and C9 finally form a membrane attack complex (Müller-Eberhard 1985; Ricklin *et al.* 2010). The membrane attack complex is responsible for attacking bacterial cells and terminating the pathway (Müller-Eberhard 1985).

The alternative pathway follows a similar cascade to the classical. However, in this pathway, complement Factor B (fB) binds to C3 and is cleaved by Factor D (fD) to form C3b (Müller-Eberhard 1985; Ricklin *et al.* 2010). The host cell easily inactivates C3b to prevent self-attack, but on bacterial cells C3b remains active and fB initiates the alternative complement cascade (Ricklin *et al.* 2010). A lectin-like molecule called properdin can help initiate this process in humans, but may not have an analogue in marine organisms (Spitzer *et al.* 2007). With fD present, C3B and fB form the C3bBb complex, which acts as a C3 convertase to form C3bBb3b (Ricklin *et al.* 2010). Finally, C3bBb3b acts as a C5 convertase to form the membrane attack complex, at which point the alternative pathway resembles the classical (Müller-Eberhard 1985).

In the lectin pathway, Mannose-Binding Lectin (MBL) and ficolins seek out carbohydrate-rich glycans on target cells in a form of lectin-glycan recognition (described below) (Ricklin *et al.* 2010). MBL (also known as millectin in corals) binds to *Symbiodinium* as well as a variety of bacteria and mediates symbiont uptake (Kvennefors

et al. 2008). MBL-Associated Serine Proteases (MASPs) help MBL and ficolins to initiate the cascade (Chen and Wallis 2004). MASP-2 cleaves both C2 and C4 to generate C3 convertase and continue as in the classical pathway towards formation of the membrane attack complex. Meanwhile, MASP-1 only cleaves C2, amplifying but not initiating the lectin pathway.

Certain complement system components allow self-regulation, preventing attack of host cells while simultaneously amplifying the anti-pathogen response. Factor H is the most important factor to protect "self" cells (Lachmann and Müller-Eberhard 1968; Thurman and Holers 2006). It prevents fB from binding to C3b, thus inactivating C3b to prevent continuation of the alternative cascade (Thurman and Holers 2006). Since C3b causes a positive feedback loop in the alternative pathway, Factor H prevents amplification of the response towards the host cell. However, Factor H also acts as a cofactor to Factor I, which cleaves C3b to increase complement activation and amplify the response towards pathogens (Lachmann and Müller-Eberhard 1968). Finally, C3b can also amplify the pathway by changing fB to allow fD to cleave fB (Thurman and Holers 2006).

A number of complement-related proteins from the alternative and lectin pathways have been characterized in invertebrates (Castillo *et al.* 2009). Proteins have been found which resemble MASP (Fujita *et al.* 2004), C3 (Castillo *et al.* 2009; Clow *et al.* 2004; Fujita 2002; Suzuki *et al.* 2002), fB (Kimura *et al.* 2009; Yoshizaki *et al.* 2005), MBL (Fujita *et al.* 2004; Kvennefors *et al.* 2008), and ficolins (Baumgarten *et al.* 2015; Skjoedt *et al.* 2010). Unfortunately, most of these genes are not well conserved between invertebrates and mammals, making functional studies more difficult (Castillo *et al.*

2009). Therefore, characterizations are based largely on presence of functional domains although further characterization has been attempted for C3 at least.

Lack of conservation and characterization for most complement genes hinders experiments that attempt to go beyond bioinformatic genome studies. Despite this, researchers have studied *in situ* complement gene expression in model marine symbioses (Castillo *et al.* 2009; Collins *et al.* 2012; Kimura *et al.* 2009; McAnulty and Nyholm 2016; Poole *et al.* 2016; Schleicher *et al.* 2014). In *N. vectensis*, complement gene expression is localized to gastrodermal tissue, where symbionts reside (Kimura *et al.* 2009). In other organisms such as *E. scolopes,* complement genes including C3 are expressed throughout the organism but especially upregulated in epithelial tissue (Castillo *et al.* 2009). *E. scolopes* complement factors are also differentially expressed in immune cells called hemocytes depending on bacterial colonization state, indicating a role for complement factors in symbiosis (Collins *et al.* 2012; Schleicher *et al.* 2014). These hemocytes are localized to the symbiont-colonized light organ and reproductive accessory nidamental gland (ANG), so complement factors may be part of symbiont-host communication in *E. scolopes* (McAnulty and Nyholm 2016). Furthermore, Poole *et al.* (2016) found that in *A. pallida*, a Factor B gene (AP Bf-1, accession # KU747967) and a MASP gene (accession # KU747969) were significantly upregulated at the onset of symbiosis in the presence of light. They also found that AP Bf-1 was upregulated on pathogen challenge, so Bf_1 appears to be involved in both symbiont and pathogen recognition in *A. pallida*. In contrast, another factor B gene, Ap_Bf-2b (accession # KU747968), was downregulated at onset and maintenance of symbiosis and at pathogen challenge. However, Ap_Bf-2b expression levels recovered after 72 hours.

3.3.2 Microbe-associated Molecular Patterns and Pathogen Recognition Receptors

Marine organisms can use PRRs to recognize MAMPS (also known as Pathogen-associated Molecular Patterns, or PAMPs) on pathogenic bacterial cell surfaces (McAnulty and Nyholm 2016). PRRs and MAMPs also mediate recognition between hosts and their symbiotic partners, enabling recognition of mutualistic symbionts and exclusion of non-mutualistic (potentially pathogenic) microbes. MAMPs may consist of peptidoglycan, lipopolysaccharide (LPS), outer membrane proteins, fimbriae, or bacterial flagellar proteins in various microbes. PRRs are immune proteins produced to recognize microbial invaders, and consist of multiple receptor families including C-type lectin-like receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and Toll-like receptors (TLRs) (Mahla *et al.* 2013). Each class of PRR, on detecting a MAMP, initiates a signal cascade to activate transcription factors, ultimately leading to opsonization of foreign microbes.

PRRs are one of the molecular signals hosts can use to recognize symbionts (Kilpatrick 2002). In *E. scolopes, V. fischeri,* produces several distinct MAMPs – LPS, outer membrane vesicles (OMVs), and tracheal cytotoxin (TCT) – which allow *V. fischeri* but no other bacteria to migrate into host crypts and colonize the light organ (Aschtgen *et al.* 2016; Foster *et al.* 2000; Koropatnick *et al.* 2004). Some *V. fischeri* MAMPS trigger and are necessary for light organ development, indicating they serve as a form of essential communication from symbiont to host (Koropatnick *et al.* 2004; McFall-Ngai and Ruby 1991). Furthermore, *E. scolopes* responds to symbionts by adjusting its PRR protein composition based on colonization state, implying that different

PRRs are needed to initiate and maintain symbiosis. For example, the PRR Galectin is more abundant in established symbiosis while *Es*PGRP5 is less abundant, although both PRRs are present at some level throughout colonization (Collins *et al.* 2012; Schleicher *et al.* 2014).

Peptidoglycan Recognition Proteins (PGRPs) are a highly conserved group of PRRs essential in innate immunity (Leulier *et al.* 2003). PGRPs contain a Peptidoglycan (PGN)-binding domain which allows recognition of and binding to bacterial cell walls (Yoshida *et al.* 1996). Although most frequently studied in insects, PGRPs are also known to defend against bacterial pathogens in bivalves (Itoh and Takahashi 2008; Martins *et al.* 2014; Ni *et al.* 2007; Wei *et al.* 2012). In insects, PGRPs induce production of antimicrobial peptides and may perform a similar function in bivalves when encountering pathogens (Détrée *et al.* 2017).

Furthermore, PGRPs are associated with maintenance of symbioses. PGRP transcripts are especially prevalent in the symbiotic mussels *Bathymodiolus azoricus* and *Mytilus galloprovincialis* (Détrée *et al.* 2017; Gerdol and Venier 2015). In several marine symbioses such as that of *E. scolopes* and the cold-seep mussel *Bathymodiolus platifrons,* PGRP expression is elevated in symbiotic tissues, particularly at induction of symbiosis (Anselme *et al.* 2006; Détrée *et al.* 2017; Nyholm *et al.* 2012; Wong *et al.* 2015). Constitutive expression of PGRPs may allow hosts to maintain communication with symbiotic bacteria and assist potential symbionts in locating symbiotic organs (Détrée *et al.* 2017; Reynolds and Rolff 2008). Although constitutively expressed, PGRPs are downregulated when the deep-sea mussel *B. azoricus* loses its symbionts, indicating a critical role for PGRPs in maintaining mutualistic relationships (Détrée *et al.* 2017).

3.3.3 Lectin/Glycan recognition

Lectins, a specific class of PRRs implicated in host-symbiont signaling, may be one way hosts can recognize symbionts by identifying highly specific molecular patterns consisting of glycans on microbial cell surfaces (Fransolet *et al.* 2012). Lectin/glycan recognition has been implicated in mutualistic associations in marine nematodes, legumes, sponges, and more (Bulgheresi *et al.* 2006; Hirsch 1999; Müller *et al.* 1981). Of interest for marine symbiosis research, sequencing has identified lectin-like sequences in the cnidarian classes Hydrozoa and Anthozoa, most prevalently in the anthozoan order Scleractinia (Hayes *et al.* 2010; Jimbo *et al.* 2010; Kvennefors *et al.* 2008; Vidal-Dupiol *et al.* 2009). Assorted lectins can bind to glycoproteins on *Symbiodinium* (Fransolet *et al.* 2012). In one experiment, coral larvae were far less efficient at establishing symbiosis when *Symbiodinium* surface glycans were removed, pointing to a role for lectin-glycan recognition in coral-*Symbiodinium* recognition (Wood-Charlson *et al.* 2006).

C-type lectins, in particular, are involved in both pathogen and symbiont recognition. For instance, mannose- and fucose-recognizing C-type lectins can bind both to the pathogenic *Vibrio coralliilyticus* and to symbiotic *Symbiodinium* spp. (Kvennefors *et al.* 2008; Stahl and Ezekowitz 1998). Millectin (MBL) is a C-type lectin found localized to nematocysts in corals and anemones which marks pathogens for destruction and may be involved in *Symbiodinium* recognition (Mohamed *et al.* 2016). It can bind to mannose, gram +/- bacteria, and *Symbiodinium,* and is upregulated in the presence of lipopolysaccharides and peptidoglycans (found on *Symbiodinium* cell surfaces) in *Acropora millepora* (Kvennefors *et al.* 2008; Kvennefors *et al.* 2010). Since

Millectin/MBL is also involved in the complement system, it may play a dual role in symbiont-host communication pathways. Other C-type lectins are known to be involved in host-parasite interactions in marine organisms, including bivalves, but not necessarily in symbioses (see Table 1 in Soudant et al. 2013).

A number of other C-type lectins are also differentially expressed in the presence of symbionts in multiple marine symbioses. Mannose Receptor 2 (MRC2) is up-regulated at four hours post-contact with *Symbiodinium* in *A. digitifera* (Mohamed *et al.* 2016). Two C-type lectins are known to be downregulated in *A. millepora* when symbionts are lost (Rodriguez-Lanetty *et al.* 2009). C-type lectins have also been identified in marine bivalve and nematode mutualisms (Bulgheresi *et al.* 2006; Gourdine and Smith-Ravin 2007). For instance, the white clam *Codakia orbicularis*, which hosts chemoautotrophic bacteria in its gills, possesses a mannose-binding C-type lectin (Gourdine and Smith-Ravin 2007). Also, a C-type lectin called Mermaid binds and agglutinates the marine nematode *Laxus oneistus's* bacterial symbionts (Bulgheresi *et al.* 2006).

Two other lectins – SLL-2 and CecL – have been implicated in symbiotic recognition (Fransolet *et al.* 2012; Jimbo *et al.* 2000; Koike *et al.* 2004). Interestingly, these lectins directly affect *Symbiodinium* physiology and provide direct evidence for host-symbiont communication (Fransolet *et al.* 2012; Jimbo *et al.* 2000). SLL-2 binds to galactose and preferentially localizes to nematocysts and *Symbiodinium* cells in the octocoral *Sinularia lochmodes* (Jimbo *et al.* 2000). In the disc coral *Ctenactis echinata* among other organisms*,* SLL-2 can induce *Symbiodinium* to transition from the flagellate motile to the coccoid non-motile form, ready for uptake by the host (Fransolet *et al.* 2012; Jimbo *et al.* 2000; Koike *et al.* 2004). In *C. echinata*, CecL also causes this

transition in *Symbiodinium*, as well as regulates rate of *Symbiodinium* cell division and limits how many symbionts are taken up by host cells (Jimbo *et al.* 2000).

According to Logan and colleagues (2010), lectin-glycan recognition may be a way for hosts to differentiate microbial strains, especially in *Symbiodinium*. They tested for microbial cell-surface glycans by using known lectins to probe for glycoproteins. In *Symbiodinium*, the lectins concanavalin A (ConA) and *Griffonia simplicifolia* isolectin (GSII) were the most commonly successful probes across all cultures. The authors furthermore state that ConA and GSII bind to mannose and N-acetyl group glycans, indicating that most *Symbiodinium* have cell-surface molecules containing these functional groups. Based on molecular probe experiments, many other glycans are present in *Symbiodinium* but are restricted to a few cultures, suggesting that glycans may be involved in host specificity (Lin *et al.* 2000; Logan *et al.* 2010; Wood-Charlson *et al.* 2006). *Symbiodinium* spp*.* glycoproteins*,* including some with α-mannose/α-glucose and α-galactose residues, are essential for symbiosis establishment in *A. pallida* and the mushroom coral *Fungia scutaria* (Lin *et al.* 2000; Wood-Charlson *et al.* 2006). When dinoflagellates are exposed to the glycoprotein-digesting enzymes trypsin, α-amylase, N-glycosidase, or O-glycosidase and then given to *Aiptasia pulchella* anemones, the anemones also fail to efficiently uptake *Symbiodinium* (Lin *et al.* 2000). Cell-surface proteins are stable throughout the *Symbiodinium* life cycle, hinting that they may be responsible for providing a long-lasting signal to host lectins for maintaining symbiosis with a specific strain of microbial partner (Logan *et al.* 2010).

3.3.4 Other possible mechanisms

Other avenues by which hosts and symbionts communicate are only beginning to be explored (Fransolet *et al.* 2012). In general, maintenance and establishment of symbiosis in marine organisms seems to involve a combination of cellular and/or molecular signals including but not limited to complement pathways and MAMPs/PRRs. For instance, in *E. scolopes,* a combination of signals between host and symbiont (the "winnowing mechanism") leads to symbiont establishment (Nyholm and McFall-Ngai 2004). In the case of *A. pulchella,* ApRab proteins regulate endocytosis of *Symbiodinium* as part of symbiosis establishment (Fransolet *et al.* 2012). One gene differentially expressed by *Symbiodinium,* H+ ATPase, is upregulated in association with symbiosis in *Tridacna crocea* and *T. maxima* larvae, as well as in the coral *Mussismilia hispida* (Kopp *et al.* 2016; Mies *et al.* 2017). However, H+ ATPase's function is to catalyze the proton pump, and its upregulation may indicate increased symbiont metabolism after colonization rather than host-symbiont communication.

Biquand *et al.* (2017) showed that *Aiptasia* spp. and coral *Symbiodinium* infection rate correlates with *Symbiodinium* cell size. The authors used both different-sized *Symbiodinium* to examine cell-size recognition and assorted-size fluorescent microspheres to control for molecular signals that might exist on *Symbiodinium* cells. A similar selection was observed for uptake of fluorescent microspheres, supporting the idea that size rather than molecular symbols is responsible for initial symbiont uptake in *Aiptasia*. On the other hand, those microspheres which were initially accepted by the host anemones were expelled within 6 days, indicating that size selection alone cannot maintain a permanent symbiosis in this case. Surface glycoproteins seem to be essential

for symbiosis maintenance, while size selection is responsible for initial uptake in *Aiptasia* spp.

3.4 Discussion

3.4.1 Known mechanisms of symbiont-host recognition

The majority of currently known symbiont-recognition mechanisms are also involved with immunity and pathogen recognition, (Weis 2008). Bioinformatics and experimental analysis have revealed major roles for both the complement system and MAMP/PRR recognition in symbiont-host communication. Other mechanisms, such as size selection and involvement of individual proteins (Biquand *et al.* 2017; Fransolet *et al.* 2012; Kopp *et al.* 2016; Mies *et al.* 2017; Nyholm and McFall-Ngai 2004), are known to be involved to some extent but may play a more minor role isolated to a few species.

3.4.2 Perspectives and future directions

Although differential expression of complement genes and roles for MAMPs and PRRs in marine symbioses have been identified, mechanistic understanding seems to be limited to these mechanisms' immune roles. Further exploration could elucidate how molecular systems of symbiont-host communication interact with symbionts. Also, some evidence exists for physiological effects of lectins on symbionts, as opposed to pathogens (Fransolet *et al.* 2012; Jimbo *et al.* 2000; Koike *et al.* 2004) and for changes in host organs caused by symbiont presence (Montgomery and McFall-Ngai 1994). Therefore, future research could examine what effects specific communication mechanisms cause in hosts and symbionts.

In the early development of giant clams, host-symbiont communication must involve specific recognition to account for species-specific symbiosis (Fitt and Trench 1981) and may involve induction of tubular system development (see Chapter 2). The complement system is likely involved in both these processes in giant clams, since complement genes are present in other Lophotrochozoans (Nonaka 2011). Whether MAMP/PRR recognition is involved in giant clam symbiosis is less certain but merits exploration. Exploring differential gene expression of these different pathways in *H. Hippopus* would help elucidate the type of effect *Symbiodinium* have on the juvenile giant clams, and perhaps exclude the possibility of increased growth effects being solely caused by nutritional products secreted by the symbionts.

4. CONCLUSION OF THE THESIS

In this research project, I found that *Symbiodinium* increase growth rates in *H. hippopus* after three weeks post-fertilization, but are not required for growth the first three weeks. I also found cell proliferation localized to the symbionts in the fourth week. These results indicate that *H. hippopus* juveniles have a facultative period of symbiont acquisition, and that symbionts start to increase growth rates and influence cell proliferation locations after this facultative period ends around day 26. These discoveries could have implications for giant clam aquaculture regarding when and how to introduce symbionts. The facultative period concept might also inform studies in other symbiotic systems, especially during the larval and juvenile phases. Also, symbiont abundance is correlated with the cell proliferation of giant clam tissues in the immediate vicinity, indicating that development of the tubular system may be stimulated by the presence of symbionts. This result implies the presence of a communication or signal between host and symbiont, which could be present in other systems as well. It also lends support to the idea that symbionts have enormous influence over a host's development, even in systems as complex as the human microbiome (Sommer and Bäckhed 2013).

Further research to complement this study could involve excluding other possible explanations and researching survival rates. Obtaining survival data for juvenile clams with and without symbionts could show more definitely whether symbionts are helpful for survival, not just growth. A light-dark experiment over the course of a day or so could reveal whether photosynthetic products from the *Symbiodinium* are aiding *H. Hippopus* growth. Also, adjusting microalgae concentrations and symbiont dose could reveal whether thresholds exist for symbiont dose and feeding.

In complement to this study, I reviewed mechanisms of host-symbiont communication, which include pathogen-recognition pathways such as the complement system and Microbe-associated Molecular Patterns (MAMPs) detected by Pattern Recognition Receptors (PRRs). Mutualistic symbionts appear to take advantage of host pathogen defense systems already in place, or may have acquired methods of evading specific host defenses to establish themselves in mutualistic relationships. Similar mechanisms may be involved in the relationship between probiotic bacteria and hosts. In scallops and oysters, introducing probiotic bacteria can increase larval survival (Balcázar et al. 2006). The increased survival may be due to nutrition benefits or reduction of infection, but the host-bacterial relationship may also involve molecular signaling mechanisms via extracellular substances produced by the bacteria (Kesarcodi-Watson et al. 2007).

Decoding host-symbiont communication could allow researchers to manipulate symbiotic relationships. In clams and corals, mechanistic understanding might lead to techniques for protecting against the damaging effects of reef bleaching or for improving aquaculture. In humans, it might be a step towards controlling obesity, asthma and other microbiome-affected maladies. In giant clams, I now know that symbionts are facultative for the first three weeks of development but start to affect host growth rates and cell proliferation by week four. The mechanisms behind these effects remain unknown, but three pathways implicated in host-symbiont recognition in other organisms provide clues to discovering the mechanisms. Host-mutualist communication in giant clams may involve MAMPs/PRRs as in *E. scolopes* and corals, or the complement system as in *E. scolopes* and sea anemones. Mutualistic symbionts in other organisms are likely to be

communicating by similar pathways. As we learn more about these pathways and about symbioses in marine organisms, we can begin to tease apart the riddle of how symbionts affect host development in species from clams to humans. Someday, these insights may even lead to cures for diseases.

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TABLES

Table 1. Summary of known mechanisms of symbiont-host recognition in marine organisms. Abbreviations are as follows: LPS, Lipopolysaccharide; MAMP, Microbe-Associated Molecular Patterns; MASP, Mannose-Binding Lectin-Associated Serine Protease; MBL, Mannose-Binding Lectin; PGRP, Peptidoglycan Recognition Protein; PRR, Pattern Recognition Receptor; and TCT, Tracheal Cytotoxin.

Mechanism	Host factors	Symbiont factors	Marine organism examples
Complement System	assorted	Glycans, other cell surface proteins	*Aiptasia pallida, Carcinoscorpius rotundicauda, Ciona intestinalis, Euprymna scolopes,* and *Nematostella vectensis* (Castillo *et al.* 2009; Collins *et al.* 2012; Kimura *et al.* 2009; Nonaka 2011; Poole *et al.* 2016; Schleicher *et al.* 2014)
(Classical)	C1q	-	-
(Alternative)	Factor B, properdin	-	*A. pallida, C. rotundicauda, C. intestinalis,* and *N. vectensis* (Nonaka 2011; Poole *et al.* 2016)
(Lectin)	MBL, ficolins		*Acropora millepora* (Kvennefors *et al.* 2008)

MAMP/PRR recognition	PGRPs (Anselme *et al.* 2006)	Peptidoglycans	*Argopecten irradians, Bathymodiolus azoricus, Bathymodiolus platifrons, Crassostrea gigas, E. scolopes, Mytilus galloprovincialis* and *Solen grandis* (Gerdol and Venier 2015; Itoh and Takahashi 2008; Martins *et al.* 2014; Ni *et al.* 2007; Nyholm and McFall-Ngai 2004; Wei *et al.* 2012; Wong *et al.* 2015)
	C-type lectin-like receptors	Mannose, fucose, LPS	*Acropora digitifera, A. millepora, Codakia orbicularis,* and *Laxus oneistus* (Bulgheresi *et al.* 2006; Gourdine and Smith-Ravin 2007; Mohamed *et al.* 2016; Rodriguez-Lanetty *et al.* 2009)
	SLL-2 lectin	Galactose	*Ctenactis echinata* and *Sinularia lochmodes* (Jimbo *et al.* 2010; Jimbo *et al.* 2000)
	CecL lectin	Cell-surface glycans	*C. echinate* (Jimbo *et al.* 2000)
	Other PRRs	LPS, outermembrane proteins, flagellar	*E. scolopes*

		proteins, glycans, TCT	(Aschtgen *et al.* 2016; Foster *et al.* 2000; Koike *et al.* 2004; Koropatnick *et al.* 2004)
	Other lectins, galectin	Cell-surface glycans (mannose; N-acetyl group, α-mannose/α-glucose, and α-galactose residues)	*A. pallida, A. puchelli[z], C. echinate, E. scolopes, Fungia scutaria[z], Geodia cydonium,* and *L. oneistus* (Bulgheresi *et al.* 2006; Collins *et al.* 2012; Jimbo *et al.* 2000; Lin *et al.* 2000; Müller *et al.* 1981; Schleicher *et al.* 2014; Wood-Charlson *et al.* 2006)
Size selection	-	Cell size	*Aiptasia* spp. (Biquand *et al.* 2017)
ApRab	ApRab protein	-	*Aiptasia pulchella* (Fransolet *et al.* 2012)

FIGURES
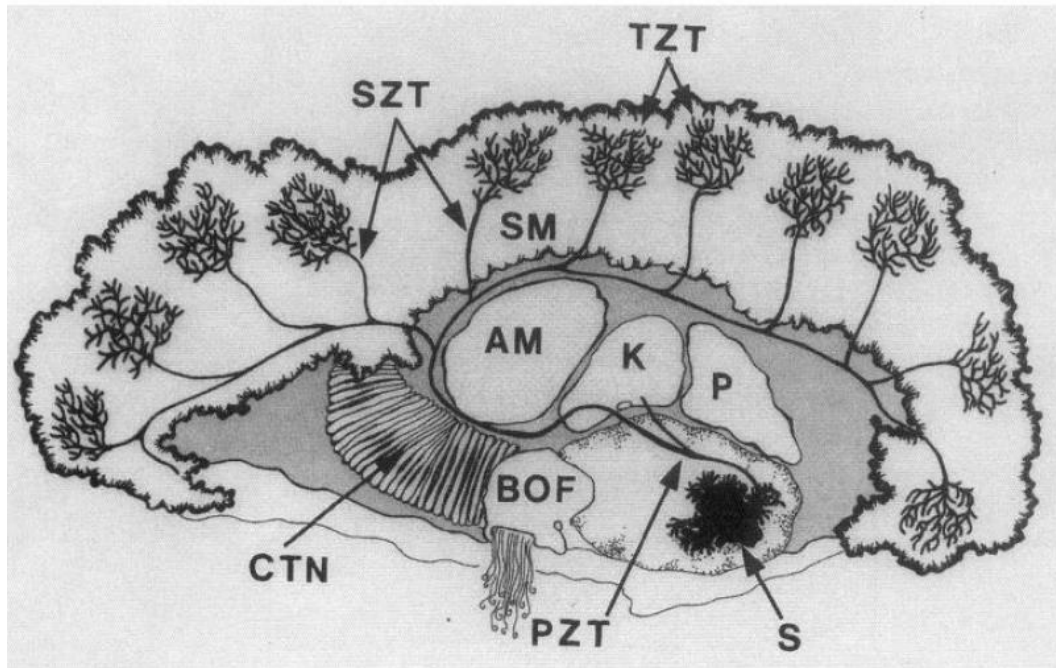
Figure 1. Illustration of the tubular system in the giant clam *Tridacna gigas* (from Norton et al. 1992).
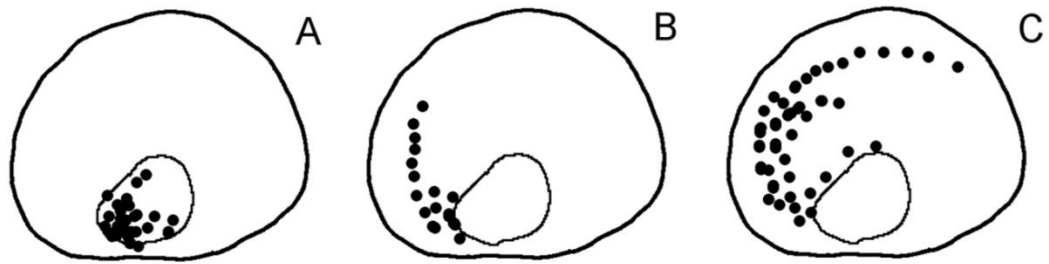
Figure 2. Representative illustrations of *Symbiodinium* colonization stages in *H. hippopus*. A. Uncolonized, B. early, and C. late stage clams are illustrated.
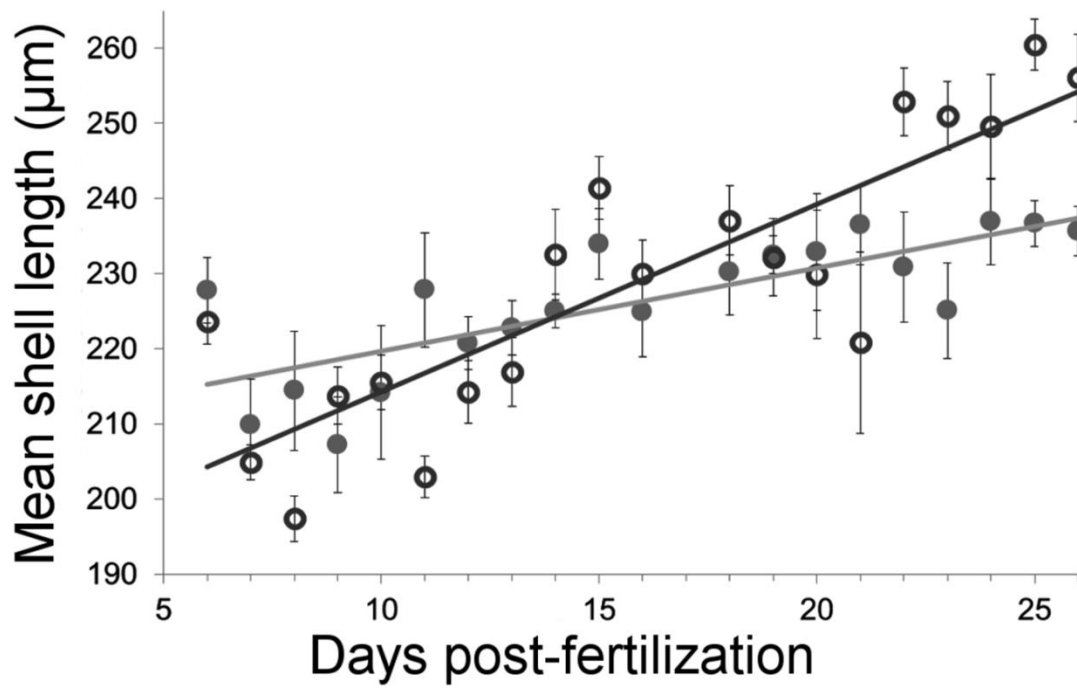
Figure 3. Shell lengths compared between the control (*closed circles*) and inoculated (*open circles*) treatments over 20 days. Error bars show one standard error.
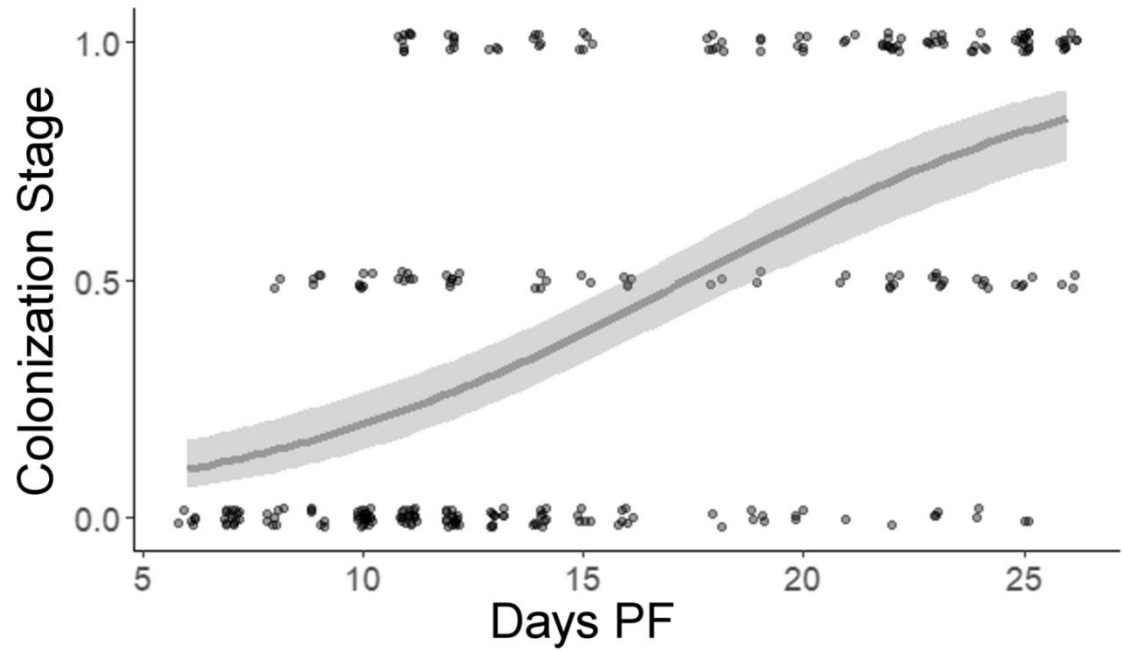
Figure 4. Logistic regression of colonization stage versus days post-fertilization to predict point of average colonization. Stages are categorized into 0) uncolonized, 0.5) early stage, and 1) late stage. Data points are jittered for clarity, and shading shows standard error.
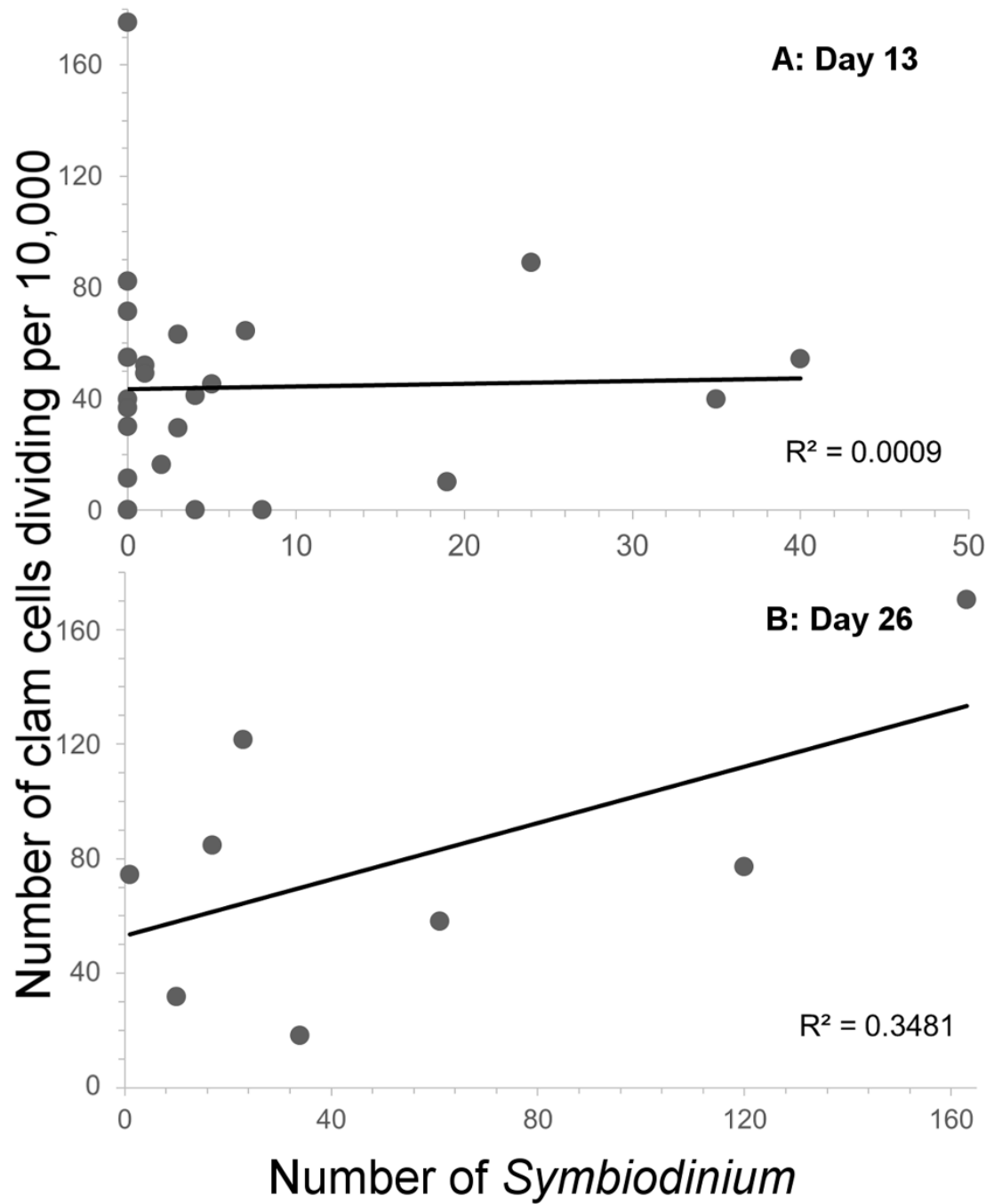
Figure 5. Proportion of EdU$^+$ clam cells over 24 h compared to number of symbionts on day 13 (A) and day 26 (B) in inoculated individuals.
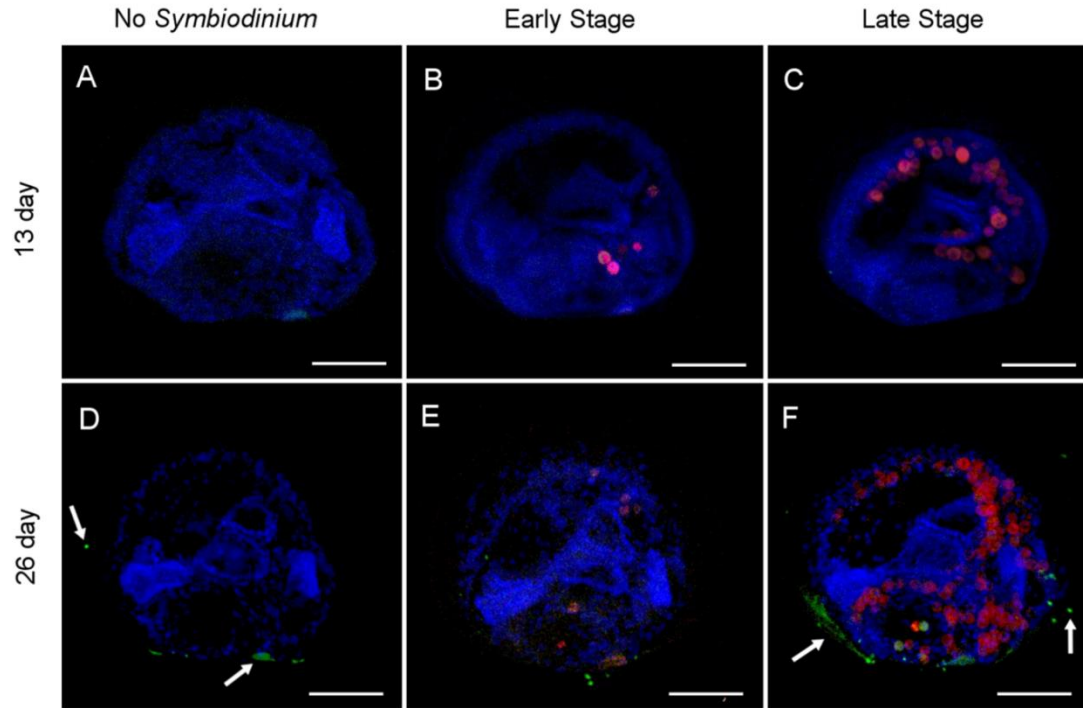
Figure 6. Confocal microscopy images showing EdU$^+$ cells (green) in *H. hippopus*, DAPI$^+$ cells (blue), and *Symbiodinium* (red). Higher numbers of EdU$^+$ cells are observed in day 26 clams (D, E, F) versus day 13 clams (A, B, C) and in late stage-symbiosis (C, F) versus early stage (B, E) or no-*Symbiodinium* (A, D) clams. EdU-staining of shell edges and hinges (*arrows*) are not dividing cells. Only EdU overlapping with DAPI stain and outside of *Symbiodinium* cells were counted as positive. Scale bar 50 μm.
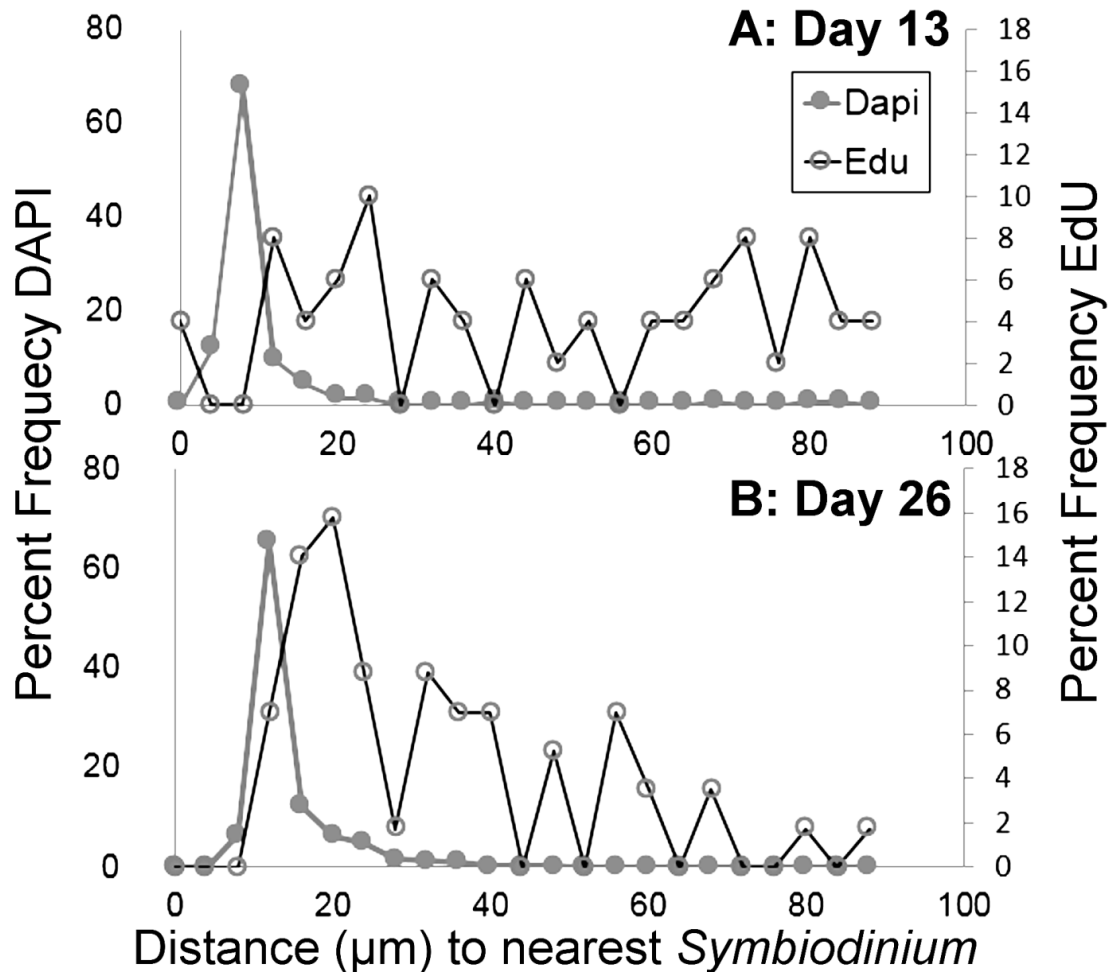
**Figure 7. Distances between the closest DAPI[+] clam cell and each *Symbiodinium* (*filled circles*) and between the closest *Symbiodinium* and each EdU[+] clam cell (*open circles*), binned to the nearest 4 μm. The distance between *Symbiodinium* and EdU+ clam cells was random on day 13 (A) and non-random on day 26 (B). On day 26, many cells were dividing near *Symbiodinium*.**
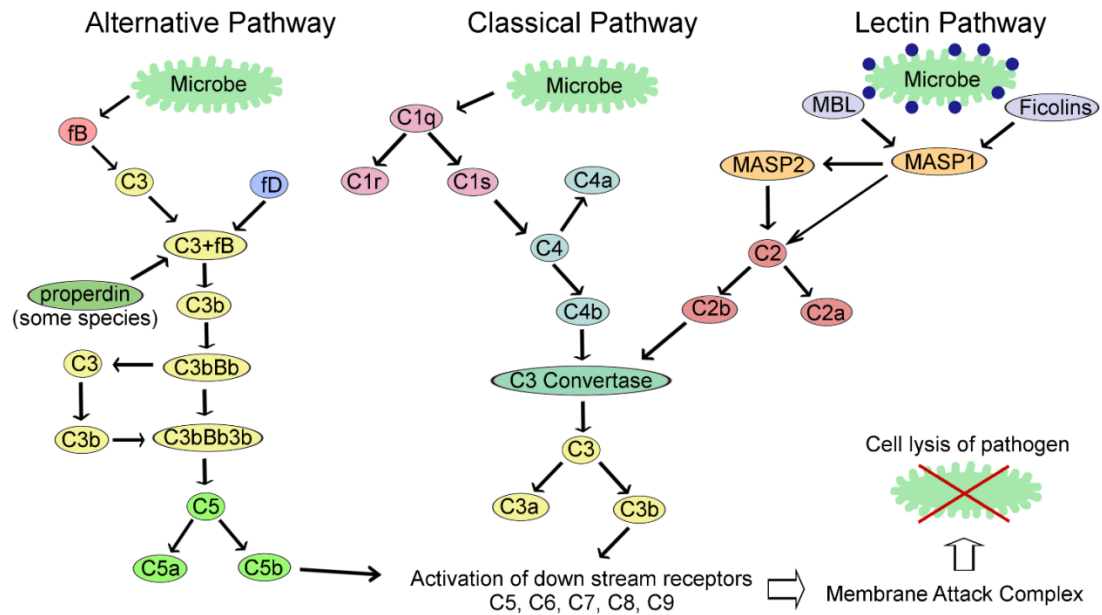
Figure 8. Three versions of the complement pathway. Thin arrows indicate activation or cleavage, while thick arrows indicate multiple factors acting together. Small dark circles represent cell-surface glycans to which MBL and ficolins bind to activate the lectin pathway. Abbreviations: fB, Factor B; fD, Factor D; MASP, MBL-Associated Serine Protease; and MBL, Mannose-Binding Lectin.

APPENDIX

Additional data for the experiment discussed in chapter 2 are presented here. Data for temperature, salinity, and pH were collected for all replicates in each treatment of *Hippopus Hippopus* juveniles. Although some variation occurred in each variable over the course of the experiment, most variation was within normal ranges for Palauan reefs. Brief drops in temperature were associated with exposure to air conditioning. Salinity (Figure A2) increased between water changes but was corrected back to ocean salinity regularly with periodic water changes. The was no relationship of pH fluctuations to temperature, salinity or any known artifacts.
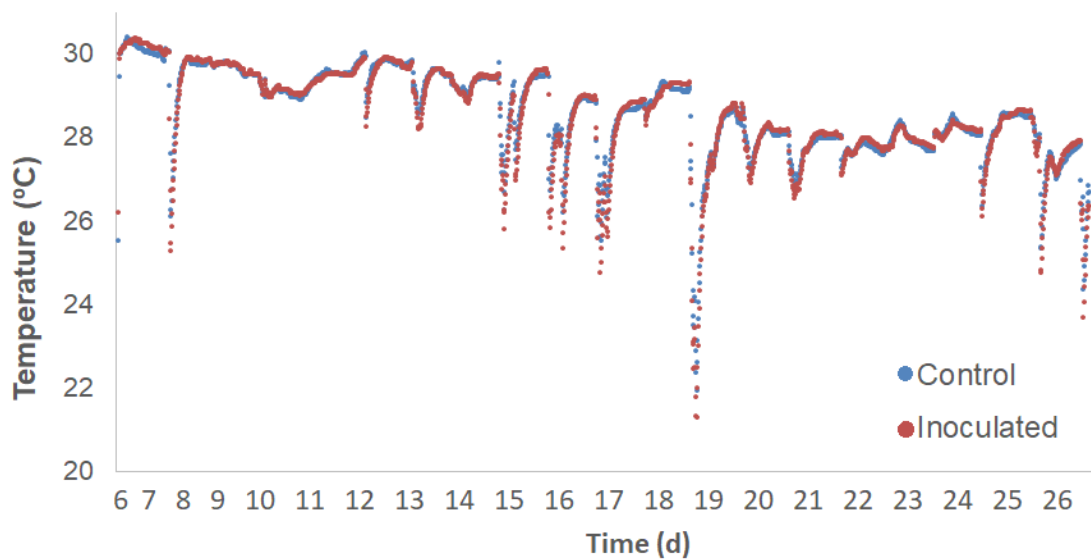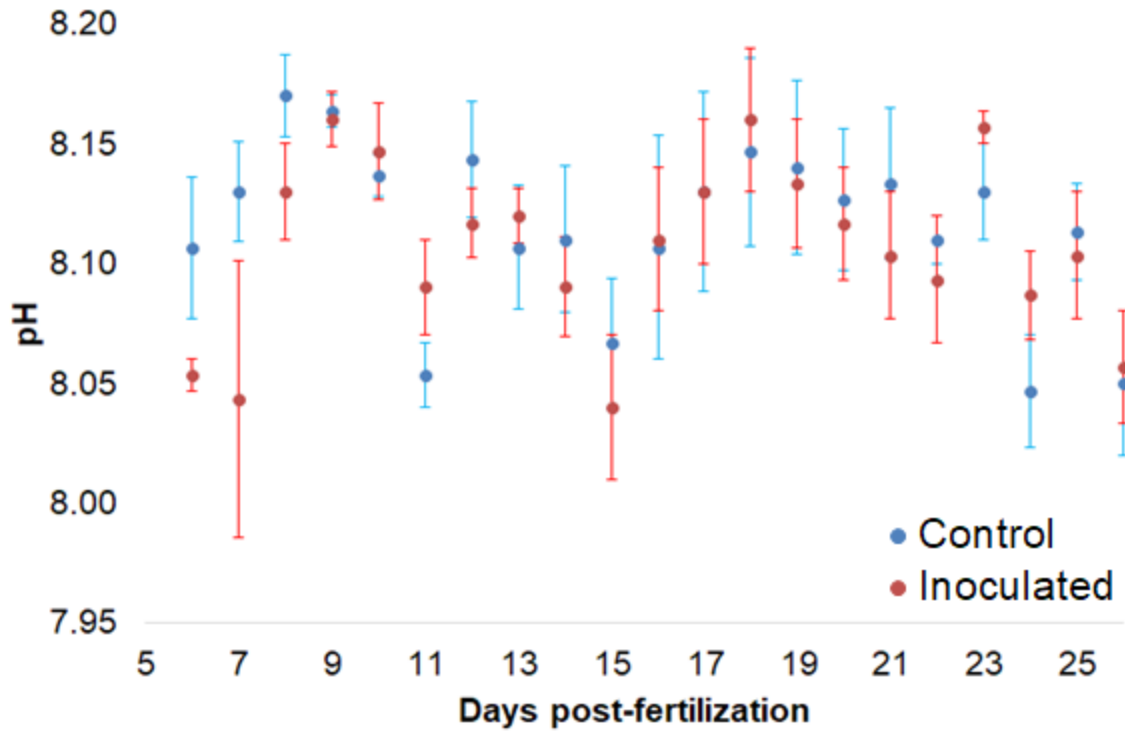

Figure A1. Temperature measurements for both treatments of giant clam larvae were logged at 15 minute intervals, and the average over all three replicate tubs for each treatment are shown. Drops in temperature followed brief exposure to air conditioning, but were short-lived and not expected to affect results as the temperature generally stayed within the 28-31ºC range typical of Palauan surface

water (Colin 2000). Control treatment, blue circles; inoculated treatment, red circles.
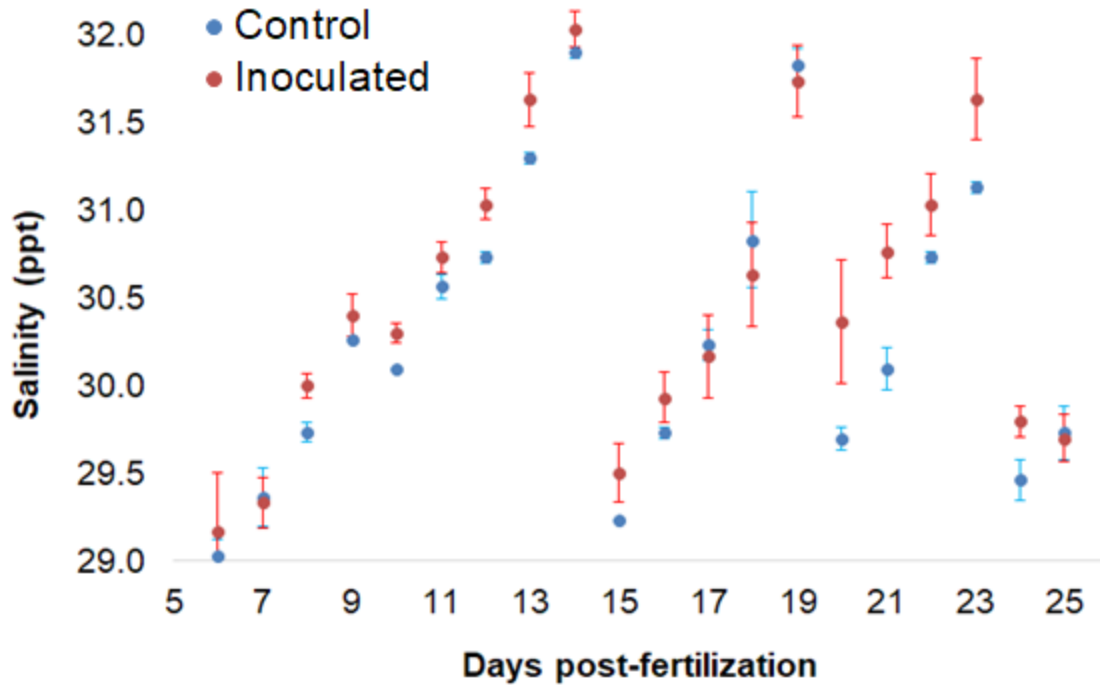
Figure A2. Salinity measurements for both treatments were logged daily in the morning and the mean over all three replicate tubs is shown with standard error. Water changes (50%) were performed on days 7, 9, 14, 19, and 23. Control group, blue circles; inoculated group, red circles. Higher salinity in the inoculated treatment on days 20-23 is likely the result of a mismeasurement during the water change on day 19.

Figure A3. Daily pH measurements for both clam treatments. The pH was logged daily in the morning and the mean over all three replicate tubs for each treatment are shown with standard error. Control treatment, blue circles; inoculated treatment, red circles.