

Regeneration in Corallimorpharia

By

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Abstract

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Regeneration in Corallimorpharia was investigated to determine mechanisms and environmental influences. *Rhodactis* sp. normal histology and regeneration following bisection over 14 days was described. Contraction of the wound occurred after 24 h and wound closure was complete as early as 4 to 8 d after fragmentation. Extension of undifferentiated tissue from the epidermis and fusion to the actinopharynx epithelium occurred, followed by differentiation of tissue layers. Structural reorganization then occurred to result in a continuous surface body wall and a cylindrical internal actinopharynx. No significant differences in regeneration rate were seen among three different species of Corallimorpharia or polyp sizes. In *Rhodactis* sp. low light (30-60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and starvation were positively associated with early regeneration. High light and feeding had a negative impact on regeneration and were accompanied by increased numbers of zooxanthellae in tissues. These findings provide the framework for future work in regeneration of corallimorpharians.

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Table of Contents

Acknowledgments	iii
Table of Contents	iv
List of Figures	vi
List of Abbreviations	xi
Chapter 1: Review of Corallimorpharia and Coral Regeneration	1
1.1 Introduction	1
1.2 Corallimorpharia.....	2
1.2.1 Taxonomy.....	2
1.2.2 Biology.....	5
1.2.3 Distribution.....	9
1.2.4 Defense	11
1.3 Regeneration	14
1.3.1 Process.....	15
1.3.2 Lesion Variation.....	18
1.3.3 Temperature	19
1.3.4 Light.....	20
1.3.5 Nutrients	23
1.4 Rationale.....	24
1.5 Hypotheses & Objectives	25
Chapter 2: Cellular Mechanisms of Regeneration in Corallimorpharia, <i>Rhodactis</i> sp.	28
2.1 Abstract.....	28
2.2 Introduction	29
2.3 Materials and Methods.....	32
2.3.1 General Conditions	32
2.3.2 Experimental Design.....	33
2.3.3 Quantitative Measurements.....	34
2.3.4 Statistical Analysis	36

2.4 Results	37
2.4.1 Normal <i>Rhodactis</i> sp. Histology	37
2.4.2 Regeneration in <i>Rhodactis</i> sp.....	46
2.4.3 Quantitative Results.....	61
2.5 Discussion.....	66
Chapter 3: Intrinsic and Extrinsic Factors That Affect Regeneration in	
Corallimorpharia.....	73
3.1 Abstract.....	73
3.2 Introduction	74
3.3 Materials and Methods.....	77
3.3.1 General Conditions	77
3.3.2 Experimental Design.....	79
3.3.3 Sampling.....	80
3.3.3.1 Regeneration Rate.....	80
3.3.3.2 Zooxanthellae Density	80
3.3.4 Statistical Analysis	81
3.4 Results	82
3.4.1 Regeneration Rate.....	82
3.4.2 Zooxanthellae Density	88
3.5 Discussion.....	94
General Discussion.....	99
References.....	104
Appendices	124
Chapter 2 Appendix	124
Chapter 3 Appendix	127

List of Figures

Figure 1.1: Competing hypotheses for relationships between Scleractinians (in bold) and Corallimorpharians (in italics) based on mt genome nucleotide sequences (A), or the amino acid sequences of the proteins they encode (B). From Lin et al. (2014)
 3

Figure 1.2: Individual polyps of *Rhodactis* sp.
 6

Figure 1.3: *Rhodactis rhodostoma* microscope squashes of marginal tentacles. (A) Filiform marginal tentacle. (B) Bulbous marginal tentacle. Scale bar = 250 µm. From Langmead & Chadwick-Furman (1999a).
 12

Figure 1.4: Nematocyst types: A- Type 1 holotrichs, B- Type 2 holotrichs, C- Microbasic-p-mastigophore, D- Type 1 microbasic-b-mastigophore, E- Type 2 microbasic-b-mastigophore. From Langmead & Chadwick-Furman (1999a).
 12

Figure 2.1: Representation of an individual polyp demonstrating where microscopic images were taken. Wound width is denoted by the double arrow. Position 1 is closest to the opening of the wound and position 3 is the furthest.
 35

Figure 2.2: *Rhodactis* sp. raised peristome (mouth) (arrow) in the center of the oral disc. There is a combination of branched discal tentacles (*) and digitiform marginal tentacles (►).
 38

Figure 2.3: Cross-section of *Rhodactis* sp. The three tissue layers are; epidermis (E), mesoglea (M), and gastrodermis (G). Mesenteries (ms) extend radially towards the center of the polyp with the mesoglea between two layers of gastrodermis; 100x H&E.
 39

Figure 2.4: Cross-section of healthy *Rhodactis* sp. A) Epidermis (E), and amoebocytes (am) within the mesoglea. Note the granular cytoplasm of the epithelial cells; cells with densely basophilic nuclei and condensed eosinophilic cytoplasm (dn), and mucocytes (mu); 1000x H&E. B) Epidermis exhibiting nematocysts in cnidocytes (cn) and eosinophilic granular structures (eg); 600x H&E. Gastrodermis (G) zooxanthellae (zx).
 41

Figure 2.5: Cross-section of *Rhodactis* sp. gastrodermis. A) Gastrodermis lines a proximal mesentery (ms) with zooxanthellae (zx), nematocysts (nm), mucocytes (mu), and eosinophilic secretory granules (gs); 400x H&E. B) Zooxanthellae within the gastrodermis; 1000x H&E. C) Mesenterial filament cnidoglandular band with a high density of nematocysts (N); 200x H&E.
..... 44

Figure 2.6: Cross-section through the actinopharynx of a healthy *Rhodactis* sp. polyp
A) Actinopharynx structure with finger-like folds of tissue lined by a specialized epithelium and mesenterial filaments present in the open space (mf); 40x H&E. B) Close up of epithelium exhibiting cilia (ci) on the surface surrounding the folds, the densely-packed epithelial cells, mucocytes, basophilic and eosinophilic granules; 400x H&E.
..... 45

Figure 2.7: Gross morphological changes in *Rhodactis* sp. A) At 2 days post-fragmentation, the polyp has contracted, bringing the cut edges together. B) 6 days: tissue development has occurred. C) 10 days: the peristome is more distinct and more central, radial symmetry is nearly attained and a small peripheral deficit at the wound site is present. D) 14 days post-fragmentation: no evidence of a wound is present. Wound area is denoted by ▲.
..... 47

Figure 2.8: *Rhodactis* sp. tissue immediately (0 h) after fragmentation. Disruption of tissue layers resulted in exposure of internal epithelia to the environment and copious sloughing of cells and debris; 400x H&E. Wound denoted by ◀.
..... 48

Figure 2.9: *Rhodactis* sp. polyps 12 h post-fragmentation. A) Cut epidermis is curling around mesoglea to reduce exposure to the environment (→). Also note a small number of necrotic cells in the epidermis, as well as the necrotic amoebocytes (▲); 400x H&E. B) Aggregation of free zooxanthellae and nematocysts (*); 200x H&E.
..... 51

Figure 2.10: Leading regenerative edges of wounded *Rhodactis* sp. tissue 1 d post-fragmentation. A) An undifferentiated mass of cells and debris extended towards the center of the wound. Necrosis was present, and adjacent to the mass all tissue layers were present, with gastrodermis organizing before epidermis; 400x H&E. B) Close up of undifferentiated cell mass; 1000x H&E. C-D) Amoebocytes within the healing margin of the mesoglea. Many amoebocytes within the mesoglea at the healing margin exhibited a reduced and condensed cytoplasm and apoptotic/necrotic nuclei; 1000x H&E.
..... 52

Figure 2.11: Two polyps of *Rhodactis* sp. 2 days post-fragmentation. A-B) The leading edge of the regenerating epidermis (E) has merged and fused (★) with the internal actinopharynx epithelium (AE). Necrosis was severe at this time; 400x H&E.

..... 54

Figure 2.12: Recently healed individual of *Rhodactis* sp. 4 days post-fragmentation. A) Previous wound site (star) and developing mesenteries (arrow); 40x H&E. B) Developing tissue has replaced the wound space. Note the prominent necrosis as the tissue is reorganized; 400x H&E. C) Mesenteries developed by invagination proximal to previous wound site; 200x H&E.

..... 56

Figure 2.13: *Rhodactis* sp. tissue 4 days post-fragmentation. A) Narrowing of the gap by proximal tissue (double arrow) while healing margin of epidermis fused to actinopharynx (arrow); 40x H&E. B) Complete fusion of epidermis with internal actinopharynx epithelium has occurred; 400x H&E. C) Necrotic cells and debris were very common in the epidermis and the tissue was nearly completely reorganized; 1000x H&E.

..... 57

Figure 2.14: *Rhodactis* sp. 8 days post-fragmentation. A) The wound is closed and all tissue layers are all present and organized; 40x H&E. B) The cellular density in new epidermis was greatly reduced; despite this all expected cells types were present and necrosis was absent; 200x H&E.

..... 59

Figure 2.15: *Rhodactis* sp. 8 days post-fragmentation demonstrating incomplete wound closure. A) The gap between the wound (denoted by a star) was greatly reduced; 40x H&E. B) Surface body wall epidermis and actinopharynx body wall epidermis were fused (*) and fully reorganized with rare necrosis/apoptosis; 400x H&E.

..... 60

Figure 2.16: Histomorphology of *Rhodactis* sp. following bisection. The mean (\pm SE) of A) zooxanthellae density, B) amoebocyte density, C) epidermal thickness, and D) mesogleal thickness were determined from three individuals at each time point. Groups that do not share the same number of asterix are significantly different ($\alpha=0.05$).

..... 63

Figure 2.17: Histomorphology of *Rhodactis* sp. following bisection. Position 1 was the measurement closest to the open wound, followed by position 2, and position 3 was furthest from the wound. The mean (\pm SE) of A) zooxanthellae density, B) amoebocyte density, C) epidermal thickness, and D) mesogaeal thickness was determined from three individuals at each time point. No significant differences among any positions at any time.

..... 64

Figure 2.18: Mean (\pm SE) decrease in *Rhodactis* sp. wound width over time after bisection on day 0. Wound width at days 2, 4, 8, and 14 d were significantly lower than at 1 d. Groups that do not share a common letter are significantly different ($\alpha=0.05$). (h=hour, d=day).

..... 65

Figure 3.1: Mean (\pm SE) change in surface area among three species of corallimorpharians at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. A) No significant difference in size was observed between large (≥ 5 cm² surface area) and small (< 5 cm² surface area) polyps (n=10 per group). B) There were no significant differences in the ability of three different species of *Corallimorpharia* to regenerate after fragmentation (n=6-8 per group).

..... 82

Figure 3.2: Mean (\pm SE) change in surface area among three species of corallimorpharians at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. Polyps regenerated significantly more tissue in the early phase than in the mid (p=0.024) and late phases (p=0.007) (n=23 per group).

..... 83

Figure 3.3: Mean (\pm SE) log change in surface area of *Rhodactis* sp. individuals at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation under different light treatments (high = 120-150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, medium= 70-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and low= 30-60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A) In the early phase, polyps in high light regenerated significantly less tissue compared to those in medium (p=0.016) and low light (p<0.001). Individuals in mid and late phases of regeneration were not affected by light treatment. B) Among light treatments, polyps under low light demonstrated a significant decline in regeneration rate over time, and under high light exhibited a peak in regeneration in the mid phase of regeneration (n=15-26 per treatment).

..... 86

Figure 3.4: Mean (\pm SE) log change in surface area of *Rhodactis* sp. individuals at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation under different feeding treatments (fed = 1mL fine powder food every 3 days or

starved). In the early phase starved individuals regenerated significantly more tissue than those that were fed ($p=0.003$). Food intake did not significantly affect the change in size in the mid and late phases of regeneration ($n=15-17$ per bar).

..... 87

Figure 3.5: Mean (\pm SE) log zooxanthellae density in small (surface area $<5\text{cm}^2$) and large (surface area $\geq 5\text{cm}^2$) polyps of *Rhodactis* sp., *Discosoma* sp., and *Ricordea yuma*. Large polyps had significantly more zooxanthellae per gram of wet tissue than small polyps ($p<0.001$) ($n=23-24$ per size).

..... 89

Figure 3.6: Mean (\pm SE) log zooxanthellae density in three different species. *Ricordea yuma* had significantly more zooxanthellae than *Discosoma* sp. ($p=0.005$) and though not significant, more than *Rhodactis* sp. ($p=0.05$) ($n=22-24$ per species).

..... 90

Figure 3.7: Mean (\pm SE) log zooxanthellae density in *Rhodactis* sp. under different light treatments (high = $120-150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and low = $30-60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) pre-fragmentation (day 0), mid-regeneration (day 7), and at the end of regeneration (day 14) post-fragmentation. At mid-regeneration, corals in high light have more zooxanthellae than those in low light ($p=0.013$). At the end of regeneration, corals under low light have more zooxanthellae than those in high light ($p=0.019$) ($n=5-8$ per bar).

..... 92

Figure 3.8: Mean (\pm SE) log zooxanthellae density in *Rhodactis* sp. under different feeding regimes; fed (1 mL of food every 3 days) and starved post-fragmentation. No significant difference was found among treatments; however, fed polyps appear to have greater zooxanthellae density ($n=4$ at time 0, and 12 at day 7 and 14).

..... 93

List of Abbreviations

ANOVA	analysis of variance
BMT	bulbous marginal tentacles
d	day
DNA	deoxyribonucleic acid
FMT	filliform marginal tentacles
FSW	filtered seawater
h	hour
H&E	haematoxylin and eosin
ITS	internal transcribed spacer
PAS	periodic acid-Schiff
ppm	parts per million
ppt	parts per thousand
PTAH	phosphotungstic acid-haematoxylin
rDNA	ribosomal deoxyribionucleic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
UV	ultra violet

Chapter 1: Review of Corallimorpharia and Coral Regeneration

1.1 General Introduction

Coral reefs are at risk worldwide with significant declines in coral cover (Bellwood et al., 2004; Bruno & Selig 2007; Gardner et al., 2003). The Caribbean has had the largest depletion in coral reefs, declining 80% in the last 30 years, from 50% coral cover to 10% (Gardner et al., 2003). Reasons for the coral cover decline include anthropogenic effects such as overfishing, or pollution, along with coral disease (Aronson & Precht 2001; Bruno & Selig 2007; Carpenter et al., 2008) and climate change (Bruno et al., 2007; Carpenter et al., 2008). Based on the International Union for Conservation of Nature Red List criteria, a high proportion of corals in the Caribbean and Coral Triangle (Indo-Pacific) are at high risk of extinction (Carpenter et al., 2008). Regeneration provides the ability to replace lost tissue and is an important survival mechanism for corals. The speed of healing or ability to regenerate sufficiently quickly may be one indicator of reef health. Regeneration directly affects healing and polyp or colony survival, and also indirectly impacts the success of sexual reproduction since colony size and health influences the production of gametes. Understanding how regeneration is affected by intrinsic and extrinsic factors can determine the factors that are most important for coral recovery. Corallimorpharians, a close relative of Scleractinia (hard corals), are likely an advantageous model to study coral regeneration. Corallimorpharians undergo rapid regeneration and propagation via asexual reproduction, are readily available, relatively robust in captivity and will provide

experimental evidence that might allow inferences about the slow growing reef-building scleractinians.

1.2 Corallimorpharia

1.2.1 Taxonomy

The order Corallimorpharia belongs to the anthozoan subclass Hexacorallia. Hexacorallia is defined by simple unbranched tentacles, paired mesenteries, and if a skeleton is present the spicules are absent in the mesoglea (Berntson et al., 1999). Hexacorallia also includes Actinaria (sea anemones) and Scleractinia (hard corals), along with more basal groups: Zoanthidea (zoanthids), Ceriantharia (tube anemones), and Antipatharia (black corals) (Daly et al., 2003). The phylogenetic position of the order Corallimorpharia has been debated for many years. It was originally proposed that Corallimorpharia are more closely related to Actinaria due to the lack of skeleton and tentacle arrangement. This theory was supported by molecular analysis using 28S ribosomal RNA sequences (Chen et al., 1995a). However, it is now accepted that Corallimorpharia are more closely related to Scleractinia, which is highly supported based on comparison of radioimmunoassay (RIA) proteins (Fautin & Lowenstein 1994), morphological features (Den Hartog 1980; Daly et al., 2003), and molecular data (Berntson et al., 1999; Medina et al., 2006; Kitahara et al., 2014; Lin et al., 2014). Another systematic conflict exists between the possibilities of Scleractinia monophyly vs. polyphyly. Some studies suggest Corallimorpharia are simply a sister group to Scleractinia, and skeletonization evolved only once (Figure 1.1 A) (Stolarski et al., 2011; Kayal et al., 2013; Lin et al., 2014). The 'naked coral hypothesis' proposes that

Scleractinia are polyphyletic and includes Corallimorpharia (Figure 1.1 B). Several authors have suggested that the order Corallimorpharia was a result of a scleractinian ancestor losing the ability to produce a skeleton during the Cretaceous period, when ocean acidification was high (Fautin & Lowenstein 1994; Medina et al., 2006; Kitahara et al., 2014). The naked coral hypothesis is strongly supported by mitochondrial amino acid/protein sequences (Medina et al., 2006; Kitahara et al., 2014) while the monophyly is supported by mitochondrial nucleotide sequences (Stolarski et al., 2011; Kayal et al., 2013; Lin et al., 2014). Therefore, the exact phylogenetic position of Corallimorpharia remains controversial.

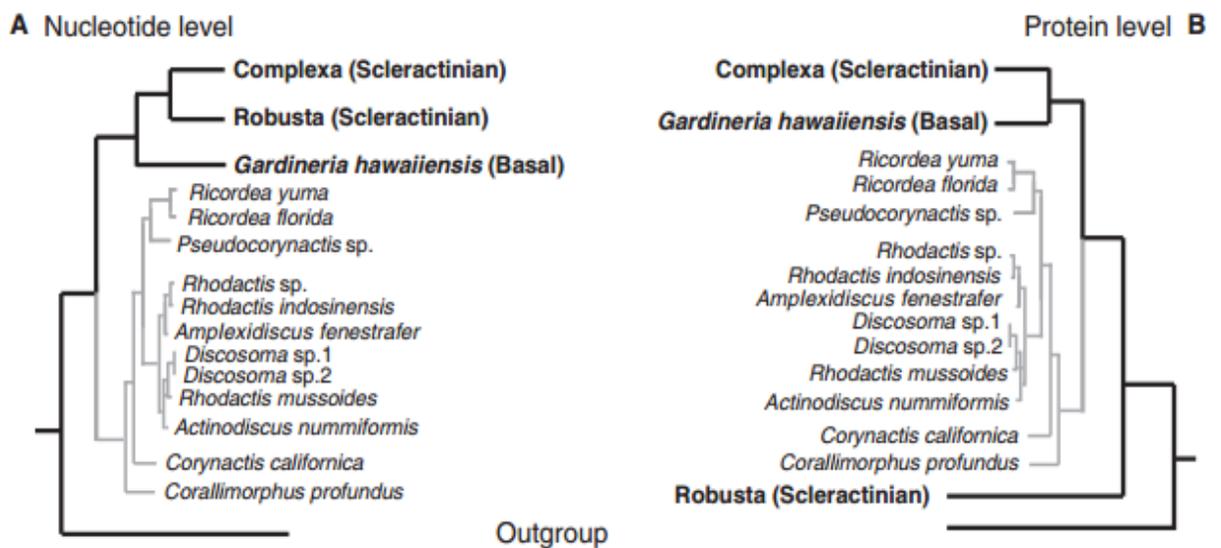


Figure 1.1: Competing hypotheses for relationships between Scleractinians (in bold) and Corallimorpharians (in italics) based on mt genome nucleotide sequences (A), or the amino acid sequences of the proteins they encode (B). From Lin et al., (2014)

Within the order Corallimorpharia, there are 4 known families: Sideractidae, Corallimorphidae, Ricordeidae, and Discosomatidae (Carlgren 1949, Den Hartog 1980). Corallimorpharia species, and sometimes genera, are very difficult to identify based on morphology and therefore it is difficult to understand their evolutionary history. The best method for determining their phylogenetic position is the use of molecular markers. Few studies have focused on the genetic relationships within genera or species in the order Corallimorpharia (Chen & Miller 1996; Chen et al., 1996; Torres-Pratts et al., 2011). It is becoming more and more customary to use ribosomal ribonucleic acid (RNA) in coral phylogenetics due to its informative level of variability (Vollmer & Palumbi 2004; Forsman et al., 2006). The internal transcribed spacer (ITS) region is a popular molecular marker for obtaining a nucleotide sequence from ribosomal deoxyribonucleic acid (rDNA). This sequence is comprised of coding sequences: 5.8S, 18S and 28S, separated by ITSs: ITS1 and ITS2. The region has been used in corallimorpharians to determine their systematic relationship (Chen & Miller 1996; Chen et al., 1996; Torres-Pratts et al., 2011). There also may be enough variation within the region to use the marker at a deeper phylogenetic level, such as population or clonal variation (Forsman et al., 2006; Torres-Pratts et al., 2011; Vollmer & Palumbi, 2004).

ITS region sequences have proven to be conserved within genera, but highly variable between genera (Chen et al., 1996). The 5.8S and 3' end of the 18S portion of the rDNA sequence are conserved within genera, and therefore make good markers to identify to a genus level (Chen & Miller 1996). For species identification, the ITS1 region was able to provide better evidence of evolutionary history than morphological studies

for four morphologically similar *Rhodactis* species from 3 different geographical locations (Chen & Miller 1996). There was a surprisingly high degree of ITS sequence conservation among geographically distinct populations of the same species, suggesting genetic exchange over long distances (Chen & Miller 1996). Lastly, *Ricordea florida* collected from 4 geographically distant sites within the Caribbean were differentiated as distinct lineages or haplotypes based on ITS sequence variation, demonstrating the utility of ITS in deep resolution of species (Torres-Pratts et al., 2011). The ITS region has therefore proven to be useful to distinguish between genera, species, and even identify haplotypes.

1.2.2 Biology

Corallimorpharia are radially symmetrical polyps that may live solitary or in colonies/aggregations (Figure 1.2). Like other anthozoans, they are composed of a “cylindrical gastrovascular cavity divided by radial septa (mesenteries) and topped with an oral disc” (Hamner & Dunn 1980). Most corallimorpharians are host to symbiotic phototrophic algal cells termed zooxanthellae, of the genus *Symbiodinium*.

Zooxanthellae are critical to the physiology of the animal as they provide the host with nearly all of their dietary needs in the form of glycerol and other organic molecules (Trench 1971; Den Hartog 1980; Muscatine 1990). There are different types of *Symbiodinium* that have been divided into “clades” (A-H), with diverse strains or species within each clade (Rowan & Powers 1991).

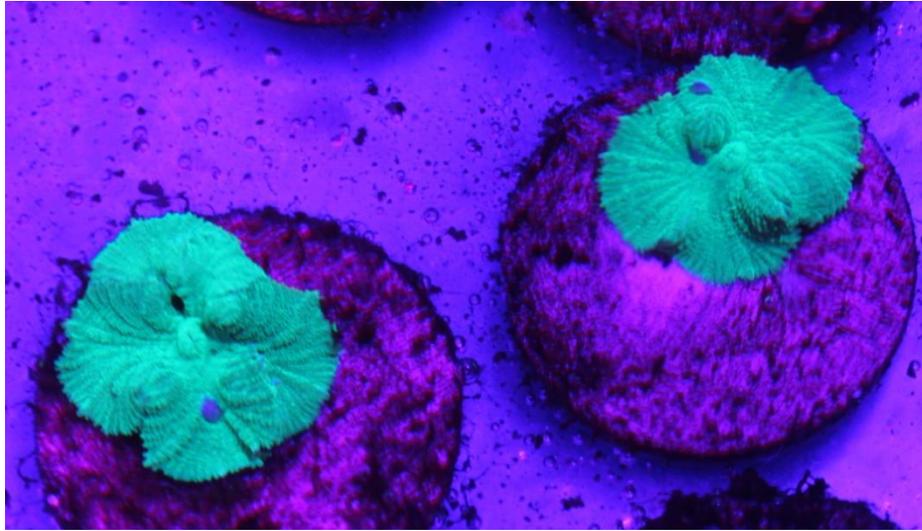


Figure 1.2: Individual polyps of *Rhodactis* sp.

Zooxanthellae in corallimorpharians are at highest density in the tentacles surrounding the margin (Elliott & Cook 1989). Their abundance in the host increases with increasing water depth, and different clades are present at varying depths and locations (Kuguru et al., 2007). Since corallimorpharians rely on their zooxanthellae for nutrients they have been noted to protect their symbionts in high light by thickening the endoderm (gastrodermis) which they reside in (Kuguru et al., 2007). Corallimorpharia may release their zooxanthellae in high temperatures; however, they are capable of recovering when temperatures return to normal, bringing zooxanthellae back into their tissues (Kuguru et al., 2007).

Corallimorpharia actively feed on plankton, detritus, crustaceans, polychaetes, and small fish, depending on polyp size, and these food sources add to the energy supplied by the symbionts (Hamner & Dunn 1980; Elliott & Cook 1989). Active feeding occurs by enveloping. Enveloping occurs by contracting radial muscle to create a bowl posture, followed by contraction of circular muscles to join the edges of the bowl

creating a cavity above the mouth (Hamner & Dunn 1980; Elliott & Cook 1989). Enveloping is cued by mechanical and chemical stimuli (Elliott & Cook 1989). The process traps food particles for the mouth to open and swallow. This behaviour has been seen in *Amplexidiscus fenestrafer* (Actinodiscidae) (Hamner & Dunn 1980) and *Rhodactis sanctithomae* (Discosomatidae) (Elliott & Cook 1989). Corallimorpharians have nematocysts located at the edges of the oral disc and during enveloping these are suspected to aid in preventing the prey from escaping (Hamner & Dunn 1980; Elliott & Cook 1989). It has been documented that active feeding occurs faster at night as polyps rest in the bowl posture as opposed to spreading out flat in the daylight to acquire light energy for their symbionts (Hamner & Dunn 1980).

Corallimorpharia occur worldwide, with many of the tropical species occurring as colonies as a result of asexual reproduction (Den Hartog 1980). Like all Cnidaria, Corallimorpharia take part in both sexual and asexual reproduction. The most common form of asexual reproduction is longitudinal fission, which occurs in larger polyps (Chen et al., 1995b; Chadwick-Furman & Spiegel 2000; Chadwick-Furman et al., 2000) by pinching and splitting across the mouth into two separate polyps (Chen et al., 1995b). However, modes of asexual reproduction are not consistent between species. For example, longitudinal fission does not occur in Ricordea (Den Hartog 1980). Other modes include inverse budding (Chen et al., 1995b; Chadwick-Furman & Spiegel 2000), two-mouth fission (Chen et al., 1995b), and marginal budding (Chadwick-Furman & Spiegel 2000). Inverse budding is achieved when a nodule of the pedal disk rises up off the substrate and is pinched off the maternal polyp (Chen et al., 1995b). Two mouth fission, a rare form only seen in *R. indosinesis*, occurs when two mouths form on the

same oral disc before pinching and separating into two individuals (Chen et al., 1995b). Lastly, marginal budding, only observed to date in *R. rhodostoma*, was accomplished by pinching of the marginal disc in one or more locations creating 2 or more polyps (Chadwick-Furman & Spiegel 2000). Asexual reproduction allows for increased population size. Doubling time of populations is 1 year in nature for *Rhodactis rhodostoma*, which is significantly faster than scleractinians (Chadwick-Furman & Spiegel 2000).

The products of asexual reproduction are aggregations of genetically identical organisms, known as clones. Clones are often assumed to be separated by 10 m, and between different clones there can be biological or morphological variation. In *Corynactis californica*, a temperate subtidal corallimorpharian, there were physiological differences between clones (Edmunds 2007). Excretion, respiration, tissue growth, and protein and energy content were all seen to vary. However, in the same study it was concluded that the differences did not alter their ability to survive different conditions in transplant experiments. In the branching coral *Madricis mirabilis*, growth rates also remained constant in a transplant experiment between two environments (Bruno & Edmunds 1997). However, when transplanted, fragments altered their phenotype to match the morphotype present in the corresponding environment. The reef-building coral *Montastrea annularis*, demonstrated significant variation in growth between separate colonies (Meesters & Bak 1994). Clonal variation is present in many corals, but the literature suggests that the variability does not affect fitness in different environments.

Asexual reproduction has been documented to slow down or stop in the months before and after sexual spawning (Chadwick-Furman et al., 2000). Timing of sexual spawning varies with species; *Rhodactis indosinesis* in Taiwan spawned in May-June (Chen et al., 1995b), while Caribbean *Rhodactis rhodostoma* spawned in June-July (Chadwick-Furman et al., 2000). Females are known to invest more in their gametes than males, and may release up to 3000 eggs (Chadwick-Furman et al., 2000). Spawning timing is thought to be a result of abiotic factors such as day length and temperature; however, additional studies are required to provide more evidence (Chen et al., 1995b; Chadwick-Furman et al., 2000).

The sex of an individual is determined by the gamete bundles' shape and colour (Chen et al., 1995b; Chen et al., 1995c). Female polyps tend to be larger than males (Chen et al., 1995b; Chen et al., 1995c; Chadwick-Furman et al., 2000), and are always located in the center of the aggregations, whereas male polyps are present along the margins with the immature polyps (Chen et al., 1995c; Chadwick-Furman et al., 2000). The position of an individual in an aggregation allows for them to allocate resources to different processes, such as reproduction in the center vs. growth, defense and stress responses along the edges. Transplantations of females to margins demonstrated that sex is extrinsically affected as they regressed in size and became males (Chen et al., 1995c). When margin polyps were moved to the center they grew and became inactive (Chen et al., 1995c). However, given more time they may become female. Sex alteration may be an adaptive strategy to maximize reproductive output and fitness.

1.2.3 Distribution

Corallimorpharians have a worldwide distribution, and the aquarium industry is based around the tropical species found in the Caribbean (Den Hartog 1980; Elliott & Cook 1989; Chen et al., 1995c) and Indo-Pacific (Chadwick-Furman & Spiegel 2000; Chadwick-Furman et al., 2000; Muhando et al., 2002; Kuguru et al. 2004). However, there are also species in the genus *Corynactis* restricted to temperate regions (Chadwick 1987; Chadwick & Adams 1991; Holts & Beauchamp 1993), as well as those in the genus *Corallimorphus* that live in temperate to polar regions in deep water (2515-3950 meters) (Fautin et al., 2009).

Research has tended to focus on tropical species because they are more accessible. Tropical corallimorpharians prefer to grow on dead coral, rock, and rubble but also grow on top of hard corals and may be fatal to the hard coral (Muhando et al., 2002). In tropical waters corallimorpharians are more abundant in shallow water (Elliott & Cook 1989; Chadwick-Furman & Spiegel 2000; Chadwick-Furman et al., 2000; Muhando et al., 2002; Kuguru et al., 2004; Kuguru et al., 2007). Most scleractinians have limited success in shallow water, where they have to face exposure to air and sunlight as well as mechanical wave damage. Corallimorpharians thrive in these conditions giving them an advantage over scleractinians. The distribution range on a reef varies among species. For example, *Actinodiscus nummiforme* and *Actinodiscus unguja* prefer the reef slope in the Red Sea (Muhando et al., 2002). Meanwhile, *Discosoma sanctithomae* and *Rhodactis rhodostoma* are unable to survive below 10 and 3 m, respectively (Elliott & Cook 1989; Chadwick-Furman & Spiegel 2000). *Ricordea*, in the Caribbean, can be found in a range of depths but below 10 m they are often solitary rather than colonial as they are at shallow depths (Den Hartog 1980). The

abundance of corallimorpharians is significantly higher in high nutrient areas with poor visibility, outcompeting hard corals that require low nutrients and clear water (Muhando et al., 2002; Kuguru et al., 2004). Corallimorpharians ranked second in “highest percent cover of living things,” next to Scleractinia in the Red Sea, and were less abundant in the marine protected areas (Muhando et al., 2002).

1.2.4 Defense

Corallimorpharia are successful, in part, because they can attack adjacent organisms in competition for space. Corallimorpharians use mesenterial filaments and acrospheres as weapons in interaction (Chadwick 1987; Miles 1991; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b). The mesenterial filaments, which are naturally used in digestion, gather in the mouth or body walls and are extruded onto the opponent after contact (Chadwick 1987). The filaments adhere to the enemy and cause tissue necrosis and death after prolonged contact (Chadwick 1987; Miles 1991; Kuguru et al., 2004). An additional mechanism for defending territory is the development of bulbous marginal tentacles (BMTs) (Figure 1.3 B). These are modified from filiform marginal tentacles (FMTs), which are naturally found in a resting non-competitive state (Figure 1.3 A). They develop by increasing in size with a thickened ectoderm (epidermis) along with an increase and switch in cnidae to type 1 holotrichs (Figure 1.4 A) (Miles 1991; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b). Type 1 holotrichs, one of many types of nematocysts, are those with elongated shafts, and spines along the thread (Langmead & Chadwick-Furman 1999a). They develop shortly after contact with other cnidarians,

and use the nematocysts to cause tissue damage (Miles 1991; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b).

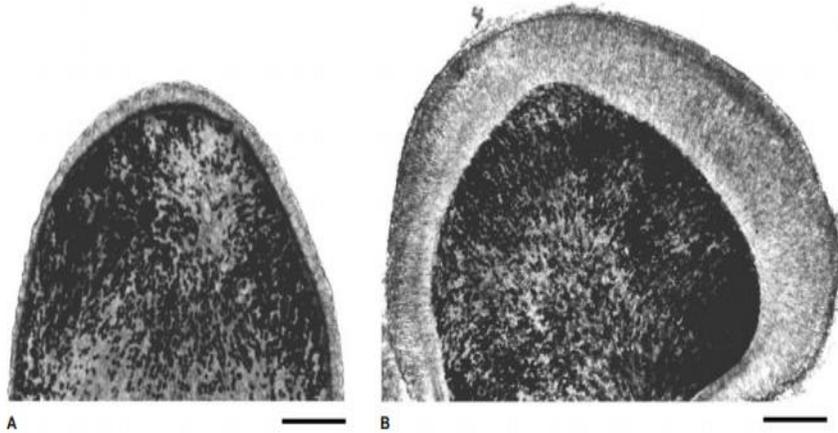


Figure 1.3: *Rhodactis rhodostoma* microscope squashes of marginal tentacles. (A) Filiform marginal tentacle. (B) Bulbous marginal tentacle. Scale bar=250 μm. From Langmead & Chadwick-Furman (1999a)

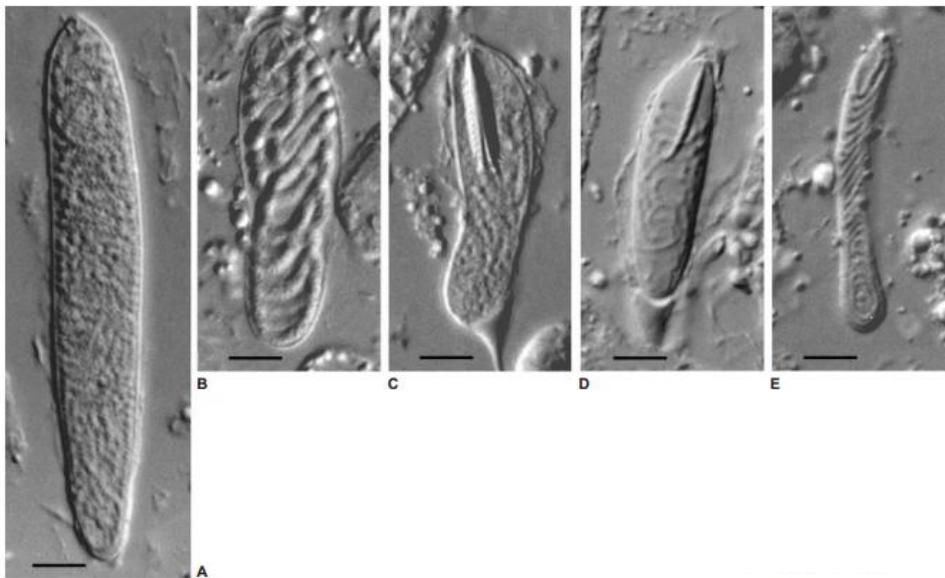


Figure 1.4:
Nematocyst types:
A- Type 1
holotrichs, B- Type
2 holotrichs, C-
Microbasic-p-
mastigophore, D-
Type 1 microbasic-

b-mastigophore, E- Type 2 microbasic-b-mastigophore. From Langmead & Chadwick-Furman (1999a)

Corallimorpharians do not display an aggressive response to all sessile neighbours. They do not respond to contact with conspecifics, algae, sponges, tunicates, and other non-Cnidaria organisms (Chadwick 1987; Miles 1991; Langmead & Chadwick-Furman 1999a). There is variation in response to different species of soft corals, stony corals and sea anemones (Chadwick 1987; Miles 1991; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b). Corallimorpharians have been described as an “intermediate competitor” (Langmead & Chadwick-Furman 1999b). When paired with branching and cup coral scleractinians, they gradually develop BMTs, damage and overgrow the competitor causing death (Chadwick 1987; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b; Kuguru et al., 2004). However, when next to massive stony corals and actinarians, there is a quicker, more intense attack pattern. This is because the corallimorpharians are first damaged by the competitor before they can develop BMTs and allow mesenterial filaments to gather (Chadwick 1987; Miles 1991; Langmead & Chadwick-Furman 1999a; Langmead & Chadwick-Furman 1999b). In order to survive the corallimorpharian must retaliate quickly. Variation in the time it takes corallimorpharians to cause fatality may take weeks for specific sea anemone competitors, or months for cup corals (Chadwick 1987).

Corallimorpharians are an intermediate competitor and don't always win. When paired with massive stony corals, such as *Meandrina meandrites* and *Platygya daedalea*, the mesenterial filaments, along with development of sweeper tentacles by

the latter, overwhelm most corallimorpharians (Miles 1991; Langmead & Chadwick-Furman 1999b). This also applied to some species of actinarians. *Metridium senile* and *Heteractis crispa* are two species that corallimorpharians cannot always outcompete (Chadwick 1987; Langmead & Chadwick-Furman 1999b). Variation in ability to compete is not only species dependent, it can be abiotically altered. In favourable environments such as those with high nutrients, corallimorpharian killing ability is increased as densities are higher and competition is more fierce (Kuguru et al., 2004). Different clones of the same species may be better competitors than others, as observed against competing *Astrangia lajollaensis* (Chadwick & Adam, 1991).

1.3 Regeneration

Regeneration is the ability to replace lost tissues. It can result in the reconstruction of a new body part, or a whole new animal from a small fragment. Regeneration is a process that resembles embryogenesis, along with asexual reproduction (Goss 1992; Alvarado 2000; Brockes & Kumar 2008). The difference between regeneration and asexual reproduction is the trigger that stimulates each process (Alvarado 2000; Chara et al., 2014). Damage is the trigger for regeneration, and a large size triggers budding. Some animals cannot reproduce asexually but are able to regenerate structures, suggesting regeneration is the basis for asexual reproduction (Carnevali 2006; Brockes & Kumar 2008). It was originally believed to be an adaptive trait that is selected for in tissues that pass a threshold of injury (Goss 1992; Bely & Nyberg 2010). This theory was dismissed as there has been no evidence

of transformation of a structure from non-regenerative to regenerative due to high levels of injury.

Two main categories of regeneration are epimorphosis, proliferation of nearby cells, or morphallaxis, migration of undifferentiated cells (Morgan 1898; Alvarado 2000; Chara et al., 2014). A close cnidarian relative, hydra, takes part in morphallaxis regeneration which may be a comparable model for corals (Agata et al., 2007; Bosch 2007). Regeneration is important to understand in corals as it is a mechanism for defense and survival for sessile organisms that are susceptible to damage and disease.

1.3.1 Process

There are limited studies on the cellular processes of regeneration in cnidarians (Patterson & Landolt 1979; Meszaros & Bigger 1999; Work & Aeby, 2010; Palmer et al., 2011) and none of which concern Corallimorpharia. In mammals, wound repair consists of four phases: clot formation, immune cell infiltration, proliferation, and maturation/remodelling. The process is somewhat conserved in species of anemones, soft corals, and hard corals (Patterson & Landolt 1979; Meszaros & Bigger 1999; Palmer et al., 2011). In *Porites cylindrica*, *Plexaurella fusifera*, and *Anthopleura elegantissima*, a plug was created of cellular debris, migrating amoebocytes, or a combination of both (Patterson & Landolt 1979; Meszaros & Bigger 1999; Palmer et al., 2011). Phagocytes ruptured at the wound surface and melanin-containing cells degranulated, releasing cellular debris and other substances that presumably contribute to plug formation (Patterson & Landolt 1979; Palmer et al., 2011). Amoebocytes aided in clot formation by creating a seal as they gathered along the healing margin, blocking

the living tissue from the external environment (Patterson & Landolt 1979; Meszaros & Bigger 1999; Palmer et al., 2011). However, coagulation is not seen in all species, as *Montipora capitata* tissues remained relatively exposed while rebuilding tissue (Work & Aeby 2010). Infiltration of immune cells was accomplished with an increase in amoebocyte density (Patterson & Landolt 1979; Meszaros & Bigger 1999; Palmer et al., 2011). Amoebocytes possess the ability to phagocytize necrotic tissue, and are the “putative immunocytes of the anthozoans” (Meszaros & Bigger 1999). They have been seen in tissues of uninjured anemones and soft corals (Patterson & Landolt 1979; Meszaros & Bigger 1999; Mullen et al., 2004; Vargas-Angel et al., 2007). Cell counts between injured and uninjured tissues demonstrated no change in overall amoebocyte numbers, only an increase at wound surface and a decrease in adjacent tissues, suggesting that amoebocytes migrate into the wound area in tissue repair in *P. fusifera* (Meszaros & Bigger 1999). The proliferation stage was not clearly defined in the Cnidaria studied, even though it was obvious that cells were populating the area as the epidermis thickened (Patterson & Landolt 1979; Meszaros & Bigger 1999; Work & Aeby 2010; Palmer et al., 2011). The increase in cell population is suggested to be due to migration from surrounding tissues, stem cells, and dedifferentiation as opposed to proliferation as evidence of mitotic figures was not apparent (Patterson & Landolt 1979; Meszaros & Bigger 1999; Work & Aeby 2010; Palmer et al., 2011). However, mitosis was observed to occur primarily at midnight in *Acropora cervicornis* (Gladfelter 1983). In Cnidarians, amoebocytes are considered to be stem cells as they have totipotent characteristics (Henry & Hart 2005; Carnevali 2006); Meszaros & Bigger (1999) thought they were precursors for epithelial cells, and Palmer et al., (2011) thought they were

precursors for melanin cells. Amoebocytes changed with time from darkly pink (H&E) stained to containing golden-brown pigment, suggestive of a melanin-containing cell precursor in *P. cylindrica* (Palmer et al., 2011). In all studies conducted thus far, after time wound size decreased, normal tissue patterns were reformed by dedifferentiation, and in one study apoptosis of amoebocytes occurred, concluding wound healing in the final phase of maturation (Patterson & Landolt 1979; Meszaros & Bigger 1999; Work & Aeby 2010; Palmer et al., 2011).

One interesting discrepancy occurs in regeneration studies between gross and histological morphology. Both *Montipora capitata* (Work & Aeby 2010) and *Pocillopora damicornis* (Rodriguez-Villalobos et al., 2016) experienced full regeneration histologically after only 12 and 8 days respectively; however, gross recovery took 40 days for both species. This is probably a result of “normal” gross morphology including pigmentation, which may take longer to recover than structural remodelling alone. Many studies only used gross observations and tended not to end the study when regeneration was complete, but rather measured the amount of repair over time for various lengths of time (Bak & Stewardvanes 1980; Meesters et al., 1997; Oren et al., 1997a; Croquer et al., 2002). The combination of lesion absence and normal tissue colour may be used to determine when regeneration is complete. However, in any regeneration study “completion” should be defined prior to experimentation to avoid confusion and to create consistency between studies.

1.3.2 Lesion Variation

The rate of regeneration is variable among scleractinians (Bak & Stewardvanes 1980; Meesters et al., 1997). The amount, shape, depth, and location of damage are major physical factors that influence regeneration rate. As the size of damaged tissue increases, the regeneration rate decreases in scleractinian models, (Bak & Stewardvanes 1980; Chadwick & Loya 1990; Meesters et al., 1997) and a maximum lesion limit that can be regenerated has been observed in some species (Chadwick & Loya 1990; Meesters et al., 1997). Along with lesion size, it's suggested that lesion shape, and in turn the ratio of perimeter to lesion surface area, is a key factor for the ability and rate of regeneration (Meesters et al., 1997; Oren et al., 1997a). With increasing perimeter:surface area ratio (P:SA), faster recovery will be obtained (Meesters et al., 1997; Oren et al., 1997a). For example, a square lesion was compared to an elongated lesion, and the square lesions, with lower P:SA, increased in size (Oren et al., 1997a). There was also variation when comparing an elongated lesion to a small single polyp lesion; elongate lesions recovered quicker regardless of how large the lesion was (Oren et al., 1997a). This is suggested to be due to the translocation of energy amongst all polyps in a colony (Oren et al., 1997a; Oren et al., 1997b). Small lesions are assumed to use local resources, whereas large lesions require energy from other areas of the colony (Oren et al., 1997a).

Two types of damage affect the regeneration rate; tissue damage vs. skeletal and tissue damage. Tissue damage recovers faster than skeletal and tissue damage in scleractinians (Bak & Stewardvanes 1980; Croquer et al., 2002). This is due to the amount of energy required to regenerate skeleton in addition to tissue. It is also

important to note that regeneration is accelerated in the initial stages of damage (Meesters et al., 1997; Oren et al., 1997a; Croquer et al., 2002) and was observed to slow over time to a similar regeneration rate regardless of lesion type or size (Meesters et al., 1997). Lastly, the location of a lesion on a colony affects the regeneration rate. Lesions inflicted towards the base of the coral colony regenerate or recover faster compared to lesions on the top (Croquer et al., 2002; Soong & Chen 2003). In summary, lesion morphology is a highly studied area when it comes to coral regeneration, and is fairly well understood.

1.3.3 Temperature

Many abiotic factors are responsible for the health of corals. Environmental factors play a role in regeneration, in addition to physical morphology of a lesion. These can include temperature, light, nutrients, and others that have been poorly studied. Temperature has been observed to have both positive and negative effects on tissue regeneration. When comparing regeneration of *Montastrea annularis* between two sites on the same island (26°C vs. 30°C) rates were increased with increased temperature (Lester & Bak 1985). However, the warmer water was a result of its proximity to a desalination plant. The effluent water may have had many additional chemical differences that were not accounted for, potentially invalidating the results.

In terms of seasons, there appears to be an optimal temperature range for regeneration. Regeneration rates were faster at a 23-25°C range compared to cool (21°C) and hot (27.5-30°C) temperatures in two separate studies (Kramarsky-Winter & Loya 2000; Denis et al., 2011). Coral growth exhibited the same pattern in Hawaiian

scleractinians in a laboratory setting (Coles & Jokiel 1977). As temperature increased, the photosynthesis:respiration rate ratio, a measurement of metabolism, decreased (Coles & Jokiel 1977). This demonstrated that the photosynthetic ability was insufficient at high temperatures to supply the host with enough organic material. In seasonal studies, other processes will likely influence regeneration, such as reproduction and radiation. The effect of temperature on coral health and regeneration is not clear. Future studies should strive to reduce confounding variables to allow a better examination of the impact of variable temperature on coral regeneration.

It is also important to note that increased temperature has a negative effect on coral growth, either slowing or stopping extension rates (Yap & Gomez 1984). Although this is not known, it could be a result of the loss of zooxanthellae in a stressed state. There is an upper temperature limit at which corals bleach by the process of expelling their zooxanthellae (Wooldridge 2010). This limit typically lies around 32°C +, and when corals have a history of being bleached, tissue regeneration is slower compared to unbleached corals (Meesters & Bak, 1994; Mascarelli & Bunkley-Williams 1999).

1.3.4 Light

Lighting (or depth) effects are also unclear relative to the effect it has on regeneration. Light is a regulator of available energy to the coral host. As mentioned previously, corals are host to symbiotic zooxanthellae that supply much of their dietary needs, and there is great diversity within the genus *Symbiodinium*. Clades or types (A-H) (Rowan & Powers 1991) may be divided further into species or strains within each clade. Some coral species host only one strain of *Symbiodinium*, whereas other species

can have individuals each housing different clades, often associated with different environments (Rowan & Powers 1991; LaJeunesse 2002; LaJeunesse et al., 2003). Some corals can even host multiple clades at a time (Rowan et al., 1997; van Oppen et al., 2001). In juvenile scleractinians, differential growth effects have been observed between clades; however, mechanisms are unknown (Little et al., 2004). These different types of symbionts have been studied in terms of genetic diversity, but the physiological differences between types are still unknown. Environmental parameters such as depth, irradiance, temperature, latitude, and longitude are all factors that may affect the acquisition of a given symbiont type in similar species of corals (Coffroth & Santos 2005). Attempts have been made to divide clades based on phenotypical features such as “sun-loving” or “shade-loving” (LaJeunesse 2002; Iglesias-Prieto et al., 2004). However, there is still some uncertainty.

In corallimorpharians, physiological adaptations to different depths include altering zooxanthellae clade, zooxanthellae and chlorophyll abundance, endodermal thickness, and dispersion of light by zooxanthellae (Kuguru et al., 2007). When switching deep water-adapted species to shallow depths, zooxanthellae clade switching occurred, and vice versa in shallow-adapted species (Kuguru et al., 2008). This information suggests symbiont type plays a role in coping physiologically with environmental changes. Photoadaptation may aid in using light in the most efficient way possible. However, in contrast, *Acropora* in environments of lower irradiance did not photoadapt the same way (Denis et al., 2013). In high light there were higher zooxanthellae densities, higher growth and reduced regeneration. There was a clear trade-off between higher growth and lower regeneration. Lower light resulted in fewer

zooxanthellae and lower lipid content, but faster regeneration. It was suggested that the high irradiance and zooxanthellae densities require more energy to maintain the symbiosis and in turn the corals have less energy for regeneration.

In contrast, it is assumed that corals at deeper depths are suspected to get less light and therefore have less energy for regeneration. Regeneration is faster in *Porites astreoides*, *Stephanocoenia michelinii*, *Acropora pulcha*, and *Montastrea* at shallow depths where light intensities are higher (Nagelkerken et al., 1999; Soong & Chen 2003; Fisher et al., 2007). However, shallow depths have a higher risk of mechanical damage due to wave action (Soong & Chen 2003). An optimal light intensity has been observed, and when out of the optimal range zooxanthellae lose pigment and carbon fixation is reduced, which in turn reduces coral growth (Coles & Jokiel 1978). Interestingly, depth had differential effects temporally in *Porites astreoides* and *Stephanocoenia michelinii* (Nagelkerken et al., 1999). Regeneration rate was faster in the first 4 weeks in the shallow water; afterwards regeneration rate became equal for colonies at 15 m and 5 m. The deeper corals then experienced a slightly faster regeneration rate than the shallow water colonies 21 and 29 days (d) post injury, respectively (Nagelkerken et al., 1999). Another temporal variation was seen in *Montastraea* spp. when shallow corals at 3 m regenerated faster than corals at all other depths after a year, but before then regeneration was not significantly different than the corals in deep water (Fisher et al., 2007). However, there have been no theories to explain temporal regeneration variation.

It is also important to note the other factors that affect the light intensity reaching a coral and in turn their growth and regeneration. Where there is high turbidity there is

often increased sedimentation. Sedimentation reduces regeneration rate in *Montastraea annularis* (Croquer et al., 2002). This may be due to the reduction in light reaching the corals, or the energy required to produce mucus to rid them of sediment (Peters & Pilson 1985; Guzman et al., 1994; Croquer et al., 2002).

1.3.5 Nutrients

Nutrients are any substances that provide nourishment for growth and survival. Corals get most of their energy requirements from zooxanthellae. Reefs occur in environments of low nutrients, and zooxanthellae allow for growth and survival. In turn corals provide the zooxanthellae with protection, CO₂, and ammonia gathered from nitrogen in the surrounding seawater, or from actively feeding. Fish excrete ammonia, and when nearby they provide more nutrients for corals to take up (Porat & Chadwick-Furman 2005; Holbrook et al., 2008). Increased ambient ammonia can allow for zooxanthellae to produce more photosynthates for the host. In both lab and field experiments, damselfish presence increased growth in the branching scleractinian *Pocillopora* (Holbrook et al., 2008). Not only do mutualistic fish increase growth, but in the giant sea anemone their presence also increased the rate of regeneration when transected (Porat & Chadwick-Furman 2005).

In contrast, nutrient compounds in the form of pollution have negative effects on growth and regeneration (Guzman et al., 1994; Renegar & Riegl 2005). High levels of nitrogen and phosphorous significantly decreased growth in *Acropora cervicornis* when added in the form of potassium nitrate (KNO₃) and monopotassium phosphate (KH₂PO₄) (Renegar & Riegl 2005). The nutrient enrichment due to the additives also

appeared to decrease zooxanthellae abundance; however, the decrease was not significant, suggesting zooxanthellae loss may not be the main cause of reduced growth (Tanaka et al., 2014). In nitrogen-enriched *Pocillopora damicornis*, zooxanthellae densities increased while lipid content decreased (Stimson & Kinzie 1991). The lower lipid levels are indicative of reduced transfer of photosynthates to the host, because the nutrient levels cannot provide for high densities of zooxanthellae. Hydrocarbons in the sediment from an oil spill also had negative effects on growth; however, interestingly; there was a positive impact on the rate of regeneration (Guzman et al., 1994). It was suggested that all energy was directed to regeneration and none was available for growth and reproduction (Guzman et al., 1994).

1.4 Rationale

Given the importance of coral reefs and the problems that they face with anthropogenic impacts and changing climate, a model organism that can be used to examine various impacts would be very useful to predict individual animal and reef recovery. The cnidarian models for studying regeneration have been easily cultured single polyps such as the hydrozoan *Hydra* spp. I proposed that a tropical species within the order Corallimorpharia may be a more suitable model, given their closer phylogenetic relationship to Scleractinia. They regenerate quickly compared to hard corals, and are single polyps that are easily maintained and acquired.

There is little research on corallimorpharians in general, and no study to date has focused on their ability to regenerate. The cellular methods by which any species of

Corallimorpharia regenerate are unknown. Like all organisms, corals have optimal environmental conditions for growth and regeneration. Changes in environmental parameters can have negative effects on the ability to regenerate lost tissue. Many studies performed to date take place in the field at sites that have variable environmental conditions. Laboratory studies are important to allow isolation of parameters before experimentation in the field where there are often confounding variables. In the case of temperature, or temperature rise, data in the literature are often plagued by confounding variables such as irradiance, day length, and pollutants. Laboratory studies are also preferred since high quality samples, for genomic or proteomic study, are more easily collected.

My proposed thesis research aimed to develop the corallimorpharian as a laboratory model organism. I described the normal histology of one species of Corallimorpharia as well as described the process by which they regenerate microscopically. In addition, I investigated the effect of intrinsic and extrinsic factors on the rate of regeneration to determine optimal conditions and polyp requirements for future work using the model.

1.5 Objectives & Hypotheses

1. To describe the normal histology of a representative species of Corallimorpharia
2. To describe the process of regeneration of a corallimorpharian species at the cellular level using histology

3. To determine a histological end point for regeneration in addition to the gross morphological end point
4. To determine, under identical conditions, which species within the order Corallimorpharia regenerates the fastest

Hypothesis 1: Given different species prefer different depths along the reef gradient, I hypothesized that those that prefer shallow areas will regenerate faster than those that prefer deeper depths due to increased light energy that allows for increased zooxanthellae, and in turn increased nutrients. If so, then *Discosoma* sp. will regenerate the fastest and have the highest zooxanthellae density followed by *Rhodactis* sp. and *Ricordea yuma*.

5. To determine, under identical conditions, which polyp size (large vs. small) regenerates the fastest

Hypothesis 2: Given that some cnidarians regenerate by morphallaxis, smaller individuals have reduced cellularity and zooxanthellae, reducing their ability to regenerate quickly. If so, then small polyps will have a slower regeneration rate and less zooxanthellae per gram of tissue than large polyps.

6. To determine which level of light intensity (high, medium, or low) will result in the fastest regeneration

Hypothesis 3: Light intensity controls zooxanthellae density, which in turn drives regeneration rate; however, there is an upper zooxanthellae density that a coral host can maintain. If so, then high light conditions will increase

zooxanthellae density and decrease regeneration rate due to high costs of maintaining symbiosis, low light conditions will decrease zooxanthellae density because of light availability and in turn decrease output for regeneration. Moderate lighting will provide for an optimal zooxanthellae density and the fastest regeneration.

7. To determine whether feeding increases the regeneration rate

Hypothesis 4: Regeneration rate is dependent, in part, on nutrient intake. If so, then increased feeding will significantly increase regeneration rate.

Chapter 2: Cellular Mechanisms of Regeneration in Corallimorpharia, *Rhodactis* sp.

2.1 Abstract

Here we describe, for the first time, the process of regeneration at a cellular level in *Rhodactis* sp. Polyps were bisected across the mouth and triplicate samples were taken at 0 and 12 h, 1, 2, 4, 8, and 14 d for routine histology. *Rhodactis* sp. regeneration is characterized by tissue extension, sealing, reorganization and differentiation, and structural reorganization. A small (120 μm) extension from the epidermis composed of undifferentiated cells migrated inwards toward the actinopharynx to wall off the exposed tissues. At the same time, the free ends of mesenteries filled the gap. The undifferentiated cell mass fused to the actinopharynx epithelia sealing off internal tissues. Tissue layers then underwent reorganization and differentiation. The epidermal edges contracted until the surface body wall became continuous providing wound closure. Once closed the mesenteries reformed pushing the actinopharynx/peristome centrally and radial symmetry was attained within 14 d after fragmentation. In addition, quantitative data including cross-sectional wound width (μm), zooxanthellae and amoebocyte density ($\text{cells}/\mu\text{m}^2$), and epidermal and mesogleal thickness (μm) were measured. The wound decreased in size after 24 h and was complete, with a continuous surface body wall as early as 4 d post-fragmentation, and complete in 100%

of polyps by 14 d. Epithelial thinning occurred 4 d post-fragmentation and amoebocyte densities peaked at 0 h and 4 d, both followed by an increase in mesogleal thickness. These findings demonstrate that *Rhodactis* sp. follows an approach somewhat similar to the scleractinians that have been studied; however, the fusion of surface body wall to the inner actinopharynx epithelia has not been described before.

2.2 Introduction

Corals are sessile organisms that are at constant risk of damage, and populations are in decline worldwide. Damage may arise from predators, competitors, disease, wave/storm action, or human impacts. To recover from damage corals, like all cnidarians, have the ability to regenerate lost tissue. Research on the mechanisms of regeneration are lacking in corals. Understanding coral biology and physiology will provide evidence as to how and whether they will recover from current stressors.

As early as the 17th century Abraham Tremblay demonstrated regeneration in the freshwater Hydrozoa, a single polyp known as a hydra or *Hydra* spp., beginning the study of regeneration in cnidarians (Dinsmore 1991). Hydra is still a widely used model organism. Hydra regeneration occurs by morphallaxis, whereby proliferation in regenerating tissue is absent but there is division of existing cells (Rowley 1902; Cummings & Bode 1984; Wittlieb et al., 2006; Bosch 2007). Endodermal and ectodermal epithelial cells in cnidarians have high plasticity and act as stem cells that self-renew and differentiate into specific cells (Wittlieb et al., 2006; Bosch 2007). Cell proliferation was necessary in both ecto- and endodermal tissues for regeneration in *Nematostella vectensis*, a small Actinaria (Passamanek & Martindale 2012). Coral

regeneration studies have focused on gross or macroscopic regeneration and the influence of different types of injury under varying environmental conditions. Understanding the cellular mechanisms by which corals recover from injury will provide a basis for future coral regeneration research.

Regeneration studies at a cellular level have been conducted on only three species of coral to date: *Plexaurella fusifera* (Meszaros & Bigger 1999), *Montipora capitata* (Work & Aeby 2010), and *Porites cylindrica* (Palmer et al., 2011). The pattern of regeneration and cells involved in coral recovery appears to be species specific. In both *P. fusifera* and *P. cylindrica*, a soft and hard coral, amoebocytes, the putative immune cells, and zooxanthellae were found to increase in numbers at the healing margin (Meszaros & Bigger 1999, Palmer et al. 2012). Amoebocytes played a major role in the regeneration process in *P. cylindrica*, sealing exposed tissue from the environment, acting as potential phagocytes, and potentially preceding differentiated cell types (Palmer et al. 2012). However, in the hard coral *M. capitata*, amoebocytes were not observed to be involved in the regeneration process (Work & Aeby 2010). In this species, regeneration of the epithelium occurred in multiple locations across the healing margin as opposed to linearly across the wound surface which was characteristic of both *P. fusifera* and *P. cylindrica* (Work & Aeby 2010, Meszaros & Bigger 1999, Palmer et al. 2012). In the most detailed study performed to date, healing in the hard coral *P. cylindrica* (Palmer et al. 2012) followed a pattern of wound repair seen in higher organisms: (i) clot formation, (ii) inflammation, (iii) proliferation, and (iv) maturation (Palmer et al. 2012).

Regeneration time varied between species; *P. fusifera* took 3 weeks to recover, and *M. capitata* 12 days until tissue maturation was complete (Meszaros & Bigger 1999, Work & Aeby 2010). As mentioned, most coral regeneration studies have focused on gross morphology, but there have been discrepancies between gross and histological data. For example, *M. capitata* took 12 days to recover histologically, but 40 days to appear fully recovered grossly (Work & Aeby 2010). In addition, *Pocillopora damicornis* experienced histological recovery at 8 days but gross recovery at 30-40 days (Rodriguez-Villalobos et al., 2016). Examination of both micro- and macroscopic regeneration should be considered when defining regeneration. Corallimorpharia are a close relative to both Actinaria and Scleractinia and have the potential to be a relevant model organism for the study of regeneration in Cnidaria. They are popular hobby subjects, are straightforward to culture, and appear to have a short regeneration period relative to scleractinians. Pilot studies on corallimorpharians suggest that individuals grossly regenerate two weeks after fragmentation. However, there are no previous scientific studies on microscopic morphology of regeneration of any species within the order Corallimorpharia.

Therefore, the purpose of this study was to describe the normal histology and the histological process of regeneration of an unidentified corallimorpharian in the genus *Rhodactis* following fragmentation. Polyps were fragmented into equal halves across the mouth and triplicate samples were taken for histology at 0 h, 12 h, 1, 2, 4, 8, and 14 days. This study will provide baseline data that will be used in the future to examine factors that influence the regeneration process.

2.3 Materials and Methods

2.3.1 General Conditions

Colonies of *Rhodactis* sp. were imported in January 2015 from Java Indonesia for the aquarium industry (Reef Wholesale, Toronto ON). Animals ranged in surface area from 0.933-5.22 cm². Corals were allowed a minimum of 2 weeks acclimation before they were removed from the rock substrate using bone cutters to chip away at the rock below polyps. Polyps were placed onto 4.5 cm diameter round ceramic discs to allow reattachment. Polyps were returned to 20.8L holding aquaria with the dimensions 40.6 x 20.3 x 25.4 cm under Radion G3 LED lighting (Ecotech Marine, Allentown PA USA) on a 8:16 h light:dark cycle. The lights were programmed to emit 100% UV, royal blue, and blue, 15% white and green, and 20% red, all at 60% brightness (Ecosmart Live). The light program was chosen based on aquarium hobby standards and corals were subject to approximately 90 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at the bottom of the aquarium (MQ-200 Quantum Light PAR Meter, Apogee Instruments, Logan UT, USA).

The system contained approximately 2500 L of re-circulating seawater prepared using synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH USA) dissolved in deionized water to achieve a salinity of 34 ± 1 ppt. Salinity was checked daily and water lost to evaporation was replenished with deionized water. Water temperature was maintained at $25 \pm 1^{\circ}\text{C}$ by a titanium plate heat exchanger (Armstrong Fluid Technology, Toronto ON). Ammonia, nitrite, and nitrate were 0-0.1, 0 and 0-15 ppm, respectively (HACH DR2800 Spectrophotometer; HACH Company, Loveland, Co USA and Master kit; Aquarium Pharmaceuticals, Chalfont, PA USA). pH was maintained at

8.2-8.3 (Accumet AB15 Plus pH meter; Thermo Fisher Scientific Inc, Ottawa ON CA) with biweekly addition of Reef buffer (Seachem, Madison GA USA). Water changes (10%) were carried out once per week. A refugium of red mangroves (*Rhizophora mangle*) and a 20 cm diameter protein skimmer (Hagen Aqualab Guelph ON) were used to maintain water conditions.

Feeding opportunities occurred once per week using *Ricordea* and *Zoanthus* marine particle food (Fauna Marin, Germany) saturated in Ultra MinD (amino acid supplement; Fauna Marin, Germany). Water flow was turned off and the food suspension was pipetted over the surface of each polyp.

2.3.2 Experimental Design

Due to the lack of published protocols for Corallimorpharia histology, a preliminary fixation trial was performed (Appendix 2.1). After acclimation three unfragmented control polyps were placed into Bouin's fixative. The remaining fifteen individuals were bisected across the mouth using a scalpel. Fragments were placed into 20.8 L aquaria on ceramic discs in the same water system with identical lighting. Fragments were identified using a random number generator and 3 replicate fragments were taken at the following time points: 0 and 12 h, 1, 2, 4, 8, and 14 d. All samples were submerged in Bouin's fixative solution (Ricca Chemical Company, Arlington TX) for 24 h, removed, rinsed with tap water and routinely processed for histology (Animal Health Lab [AHL], University of Guelph). Samples were embedded in paraffin oral side down and two 4 µm cross sections from each individual were taken 150 µm apart, and stained with hematoxylin and eosin (H&E). Additional stain procedures, including

periodic acid Schiff reagent (PAS), Masson's trichrome, Luna for eosinophilic granules, Giemsa, and phosphotungstic acid haematoxylin (PTAH) were used on healthy individuals to determine normal cellular properties.

2.3.3 Quantitative Measurements

For each polyp, the section with the actinopharynx present and with complete tissue was chosen for measurement. Three images (Olympus DP71 microscope; imaging software Olympus cellSens) were taken from the each side of the wound (Figure 2.1) at 200x magnification for each polyp. Images were imported into Image J (Abramoff et al. 2004) and were calibrated using the scale bar provided by cellSens for measurement. Epidermis and mesoglea thickness (μm) as well as zooxanthellae and amoebocyte density ($\text{cells}/\mu\text{m}^2$) were measured. For each sampling time there were three replicate polyps and 6 pictures in total from each polyp, 3 on the left and 3 on the right of the wound ($n=18$). In addition, another image was taken at 40x magnification spanning the opening of the wound, including both ends of the regenerating surface body wall. These latter photos provided a measure of the width of the wound as the smallest straight line from the left to right surface body wall for each polyp at each time point.

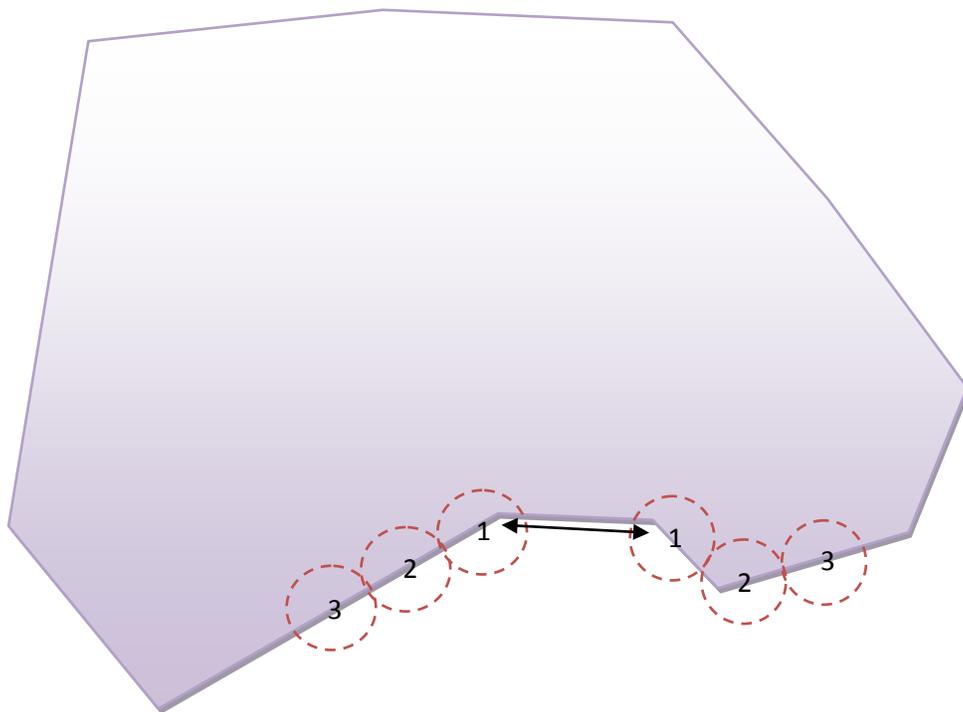


Figure 2.1: Representation of an individual polyp demonstrating where microscopic images were taken. Wound width is denoted by the double arrow. Position 1 is closest to the opening of the wound and position 3 is the furthest.

Tissue thickness was measured using pictures that included all tissue layers; epidermis, mesoglea and gastrodermis, and to ensure consistency tentacles were excluded from measurements. Three measurements at 200x magnification were taken at approximately equal distances and a mean thickness (μm) was generated. Zooxanthellae and amoebocyte density measurements were only determined at points with intact mesoglea or gastrodermis housing the cells of interest. Densities were obtained by measuring the surface area of the desired tissue, and a count of the desired cells within the outlined section was made. Zooxanthellae densities were measured within gastrodermis directly below the mesoglea and between two mesenteries. Amoebocyte densities were measured within the mesoglea where present.

2.3.4 Statistical Analysis

In SPSS (IBM Statistical Software) a multivariate general linear model/MANOVA was initially chosen for analysis. However, due to the lack of linear relationships between dependent variables, assumptions were not met for a MANOVA, and separate two-way ANOVA's for each dependent variable was conducted followed by a post hoc test when significance was observed. Variables were tested for normality (Shapiro-Wilk $p>0.05$) and if they did not pass transformations were performed until they did. They were then tested for equal variance (Levene's test $p>0.05$), and if they failed this test a Welch ANOVA was performed. When significance was found the appropriate post hoc test was then performed. The two-way ANOVA tested for differences among the measured variables over time as well as the effect of proximity to the wound (position 1-3). When the Welch test was performed the interaction of time and position could not be tested. In addition, a one-way ANOVA was performed on the wound width over time.

2.4 Results

2.4.1 Normal *Rhodactis* sp. Histology

Corallimorpharians are single-polyp species that may be solitary, or grow in aggregations. Polyps of the corallimorpharian *Rhodactis* sp. are radially symmetrical and dorso-ventrally flattened with a discoidal body shape (Figure 2.2). In the laboratory, they have been seen to reach oral disc diameter of 5.5 cm. In the center of the oral disc is a raised peristome (mouth) that leads into the tubular actinopharynx and connects to the gastrovascular cavity like scleractinian polyps (Galloway et al., 2007). They have both branched discal tentacles arranged radially, as well as digitiform marginal tentacles. There is no tentacle-free zone, tentacles are non-retractile, and lack acrospheres (Figure 2.2). The surface body wall consists of the epidermis, mesoglea, and gastrodermis (Figure 2.3). From the mesoglea are extensions to the center of the polyp called mesenteries (Figure 2.3). Mesenteries can be complete or incomplete and are lined with gastrodermis to allow for increased surface area for digestion; they provide structural support, as well as act as the location of gonadal development similar to scleractinians (Galloway et al., 2007).

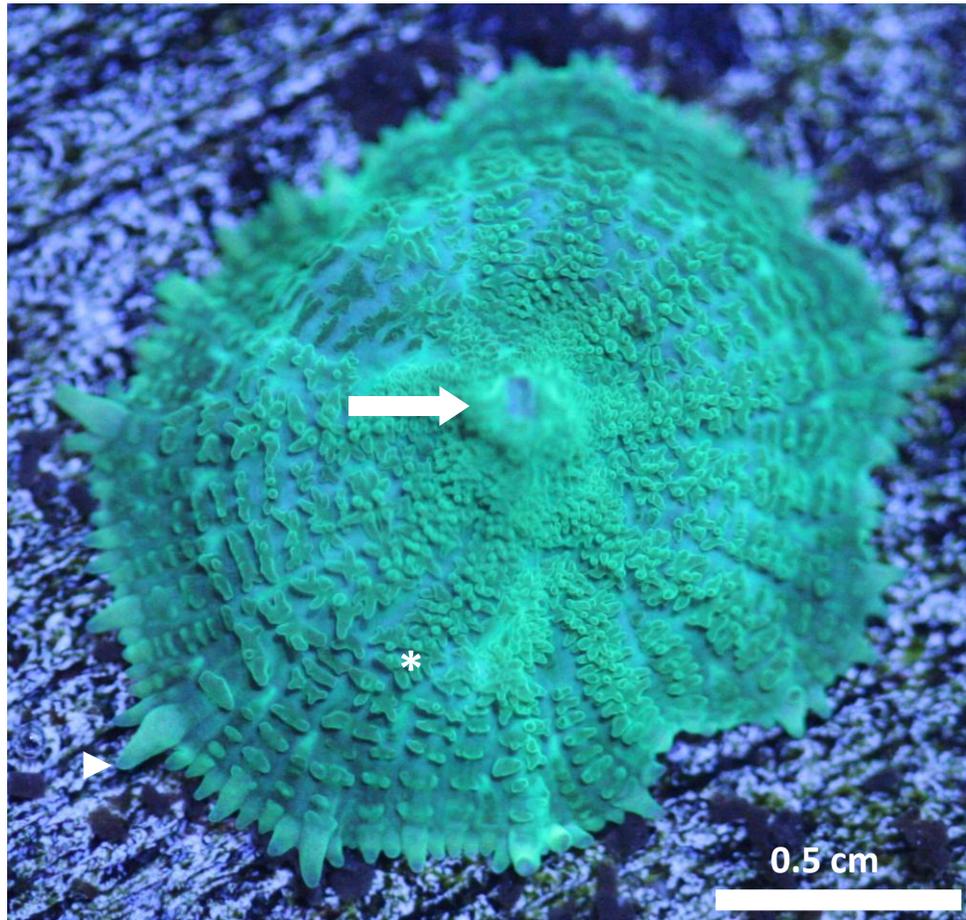


Figure 2.2: *Rhodactis* sp. raised peristome (mouth) (arrow) in the center of the oral disc. There is a combination of branched discal tentacles (*) and digitiform marginal tentacles (▶).



Figure 2.3: Cross-section of healthy *Rhodactis* sp. The three tissue layers are; epidermis (E), mesoglea (M), and gastrodermis (G). Mesenteries (ms) extend radially towards the center of the polyp with the mesoglea between two layers of gastrodermis; 100x H&E.

On average, the epidermis was 66.2 μm ($\pm 5.5 \mu\text{m}$) thick. Epidermis was pseudostratified containing columnar cells with non-discrete borders. Eosinophilic granules, 0.5-1.5 μm in diameter, were present within the cytoplasm of epithelial cells (Figure 2.4 A). Additionally, there were sparsely scattered, more intense eosinophilic granules clustering into an elongated ellipse shape up to 15 μm long at the apical epidermis (Figure 2.4 B). Clusters of brown pigmented, refractile, round to irregularly shaped granules 1-2 μm in diameter were also found sparingly throughout the epidermis at the apical surface (Figure 2.4 A). The nuclei of the epithelial cells were present in a band approximately one-third to one-half the distance from the apical surface of the epithelium toward the base. There was also a less common population of morphologically distinct, basally-oriented, cells with a much more condensed nucleus and cytoplasm. These were present at a very low density, approximately 1 per 20 epithelial cells (Figure 2.4 B). Mucocytes were present at approximately 1 to every 10 epithelial cells and were evenly spaced throughout the span of the epidermis (Figure 2.4 A). PAS did not preferentially stain the mucocytes (Appendix 2.2). Nematocysts were identified as 10-30 μm elongated eosinophilic structures along the apical surface of the epidermis that were commonly present on the surface body wall of the coral and were most common in tentacles. Nematocysts were located intracellularly within cnidocytes, and were surrounded by an intracytoplasmic space or vacuole (Figure 2.4 B).

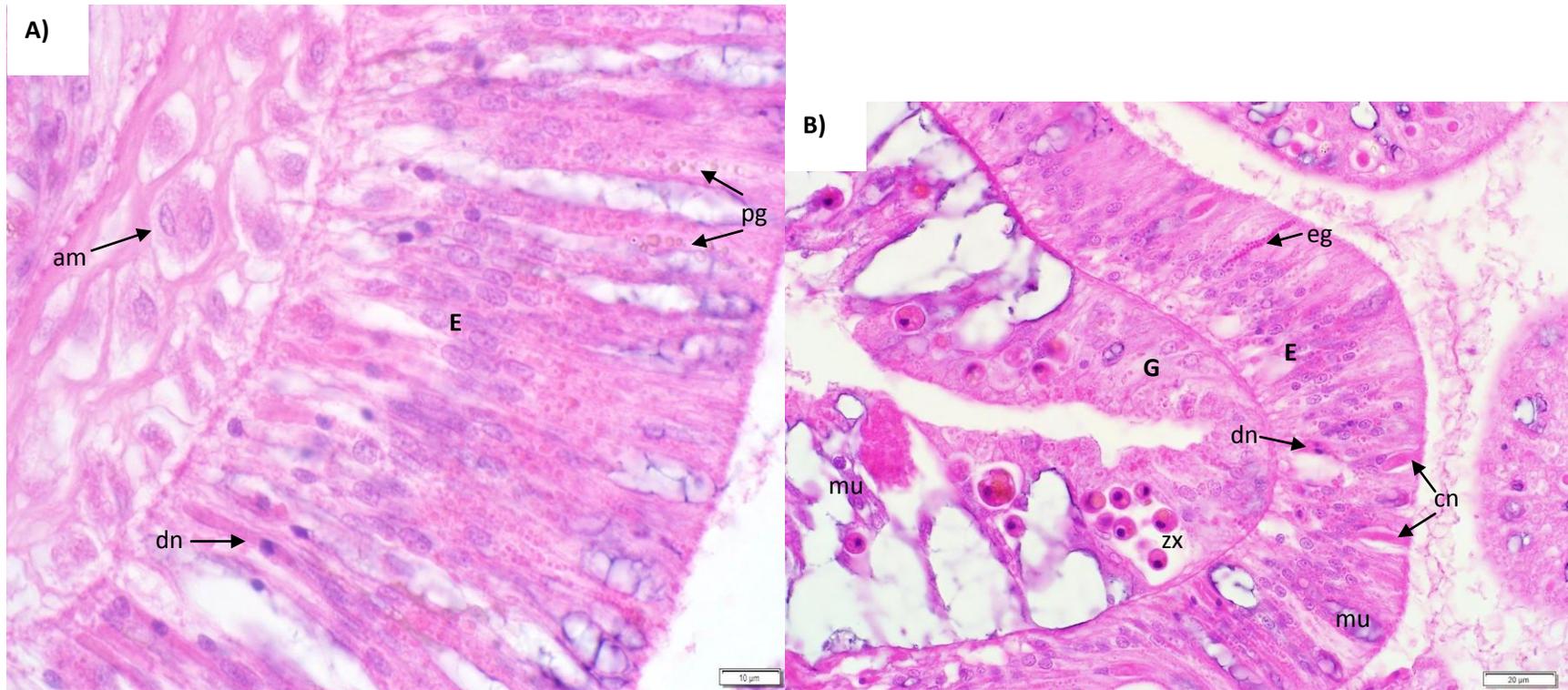


Figure 2.4: Cross-section of healthy *Rhodactis* sp. A) Epidermis (E), and amoebocytes (am) within the mesoglea. Note the granular cytoplasm of the epithelial cells; cells with densely basophilic nuclei and condensed eosinophilic cytoplasm (dn), and mucocytes (mu); 1000x H&E. B) Epidermis exhibiting nematocysts in cnidocytes (cn) and eosinophilic granular structures (eg); 600x H&E. Gastrodermis (G) Zooxanthellae (zx).

The mesoglea ranged from 5-55 μm thick and was at its' widest in tissue sections below the tentacles and also closer to the gastrovascular cavity. This layer is eosinophilic and is composed of a collagenous matrix that includes amoebocytes. Amoebocytes were on average 10-15 μm in diameter with an irregularly shaped finely condensed nucleus and a granular cytoplasm, and appeared to reside within lacunae (Figure 2.4 A). The arrangement is somewhat comparable to chondrocytes in their cartilaginous matrix (Young 2013). Amoebocytes were present at the highest densities in the thickest sections of mesoglea, either below tentacles and/or closer to the gastrovascular cavity. Amoebocytes did not preferentially stain with PAS, Luna for eosinophilic granules, Giemsa, or PTAH (Appendix 2.2).

The gastrodermis was located below the mesoglea and also lined the mesoglea of the mesenteries (Figure 2.5 A). Within this tissue layer were zooxanthellae, symbiotic algal cells, housed within vacuolated gastrodermal cells. Zooxanthellae were visible as distinct round cells 6-9 μm in diameter with a highly condensed granular eccentric nucleus (dinokaryon) and irregularly shaped brown pigmentation that occupied approximately 25 percent of the cell (Figure 2.5 A). On average, zooxanthellae were present at a density of 0.0011 (± 0.0002) cells/ μm^2 of gastrodermis. Nematocysts were also commonly found in the gastrodermis, in tentacle gastrodermis, and in the gastrodermis lining the mesenteries. Gastrodermal nematocysts were in higher abundance compared to those in the epidermis and they were present at the highest density at the free end of the mesenteries (Figure 2.5 C). Clusters of eosinophilic granules were sparsely seen throughout the apical surface of the gastrodermis (Figure

2.5 A). In some sections, brown refractile pigmented granules were less common here compared to the epidermis.

Lastly, there was a distinct gastrovascular cavity in the center of the polyp that opens to the environment at the mouth/peristome. The cavity was irregularly-round with many finger-like folds that projected into the cavity making up the actinopharynx (Figure 2.6 A). The ciliated epithelial cells were the most common cell type and were densely-packed with indistinct cell membranes (Figure 2.6 B). Mucocytes were the second most common cell type and were often large, approximately 7-12 μm in length. Intracellular clusters of both eosinophilic and basophilic granules were scattered along the epithelial apical surface as well as large nematocysts (Figure 2.6 B).

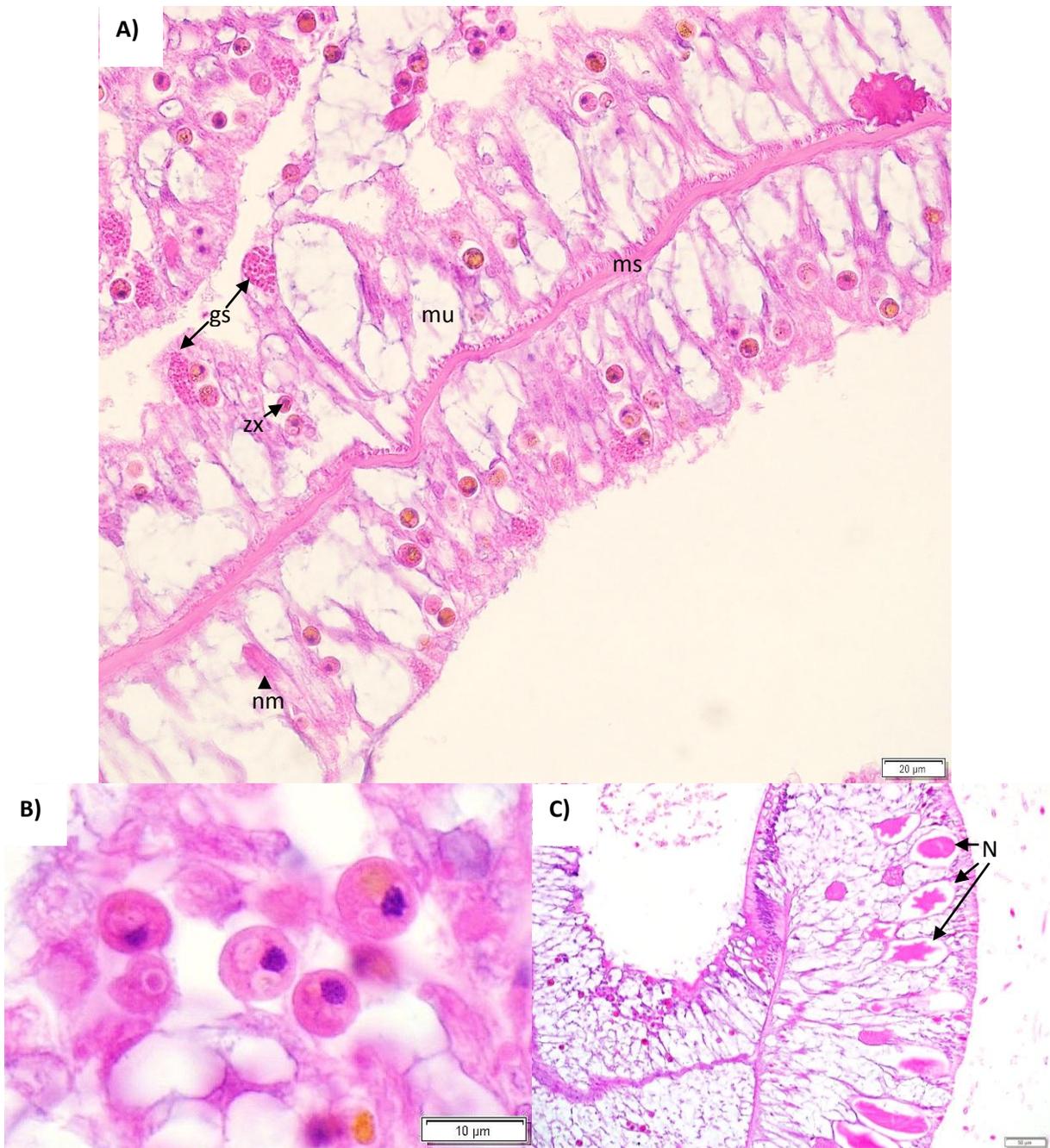


Figure 2.5: Cross-section of *Rhodactis* sp. gastrodermis. A) Gastrodermis lines a proximal mesentery (ms) with zooxanthellae (zx), nematocysts (nm), mucocytes (mu), and eosinophilic secretory granules (gs); 400x H&E. B) Zooxanthellae within the gastrodermis; 1000x H&E. C) Mesenterial filament cnidoglandular band with a high density of nematocysts (N); 200x H&E.

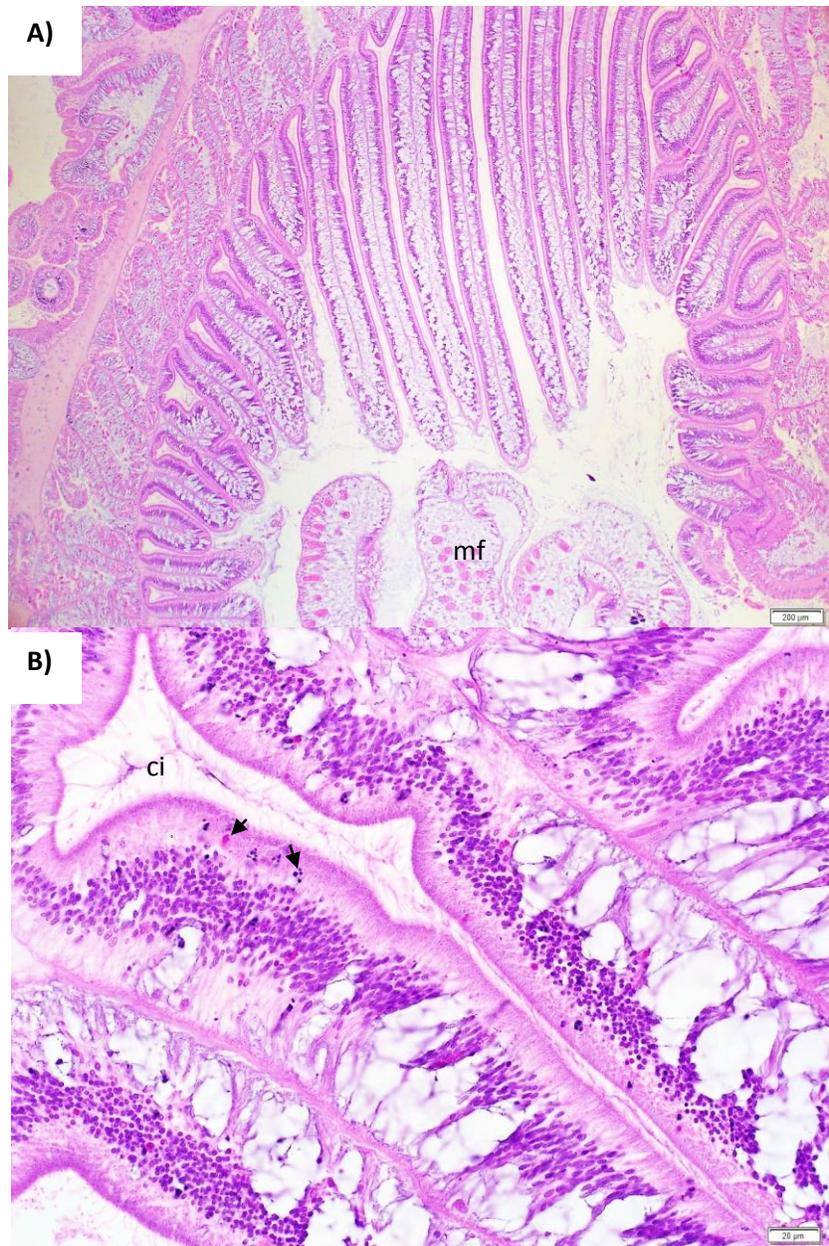


Figure 2.6: Cross-section through the actinopharynx of a healthy *Rhodactis* sp. polyp

A) Actinopharynx structure with finger-like folds of tissue lined by a specialized epithelium and mesenterial filaments present in the open space (mf); 40x H&E. B) Close up of epithelium exhibiting cilia (ci) on the surface surrounding the folds, the densely-packed epithelial cells, mucocytes, basophilic and eosinophilic granules; 400x H&E.

2.4.2 Regeneration in Rhodactis sp.

Gross morphology provides a basis for the histological description. By 2 days after transaction, the polyp had contracted in size, bringing the two cut edges closer together; however, the peristome was still at the edge of the tissue. Tissue development by day 6 resulted in the peristome having migrated part way towards the centre of the reorganized disk. By day 10 the peristome is more distinctly developed, is nearly placed centrally again, and there is a small peripheral deficit where the wound had been made. By day 14 healing and reorganization was completed (Figure 2.7).

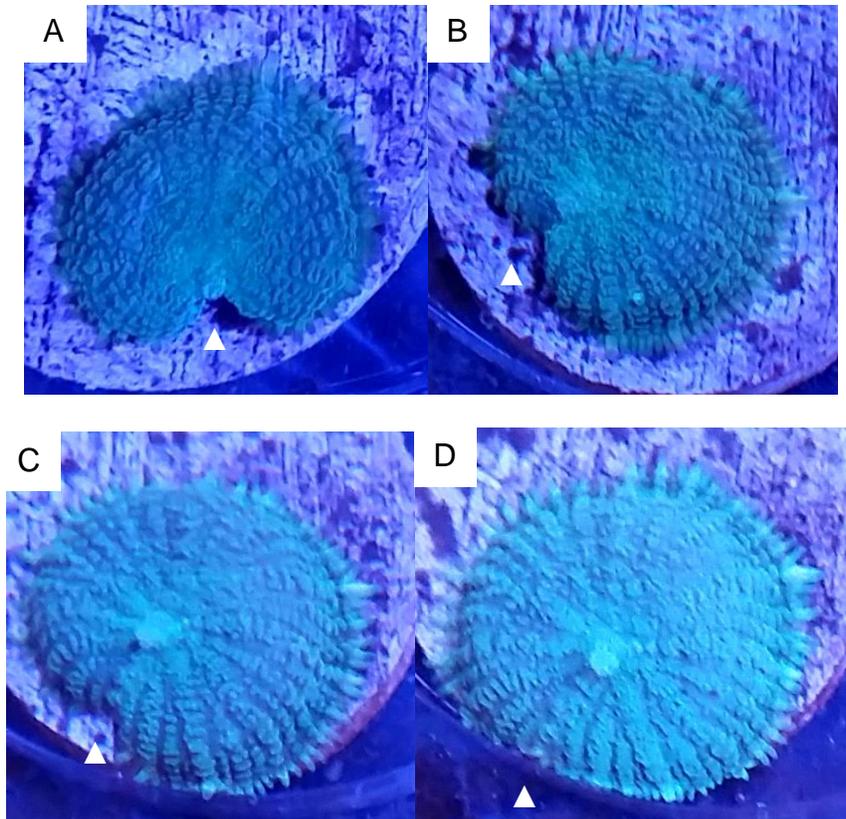


Figure 2.7: Gross morphological changes in *Rhodactis* sp. A) At 2 days post-fragmentation, the polyp has contracted, bringing the cut edges together. B) 6 days: tissue development has occurred. C) 10 days: the peristome is more distinct and more central, radial symmetry is nearly attained and a small peripheral deficit at the wound site is present. D) 14 days post-fragmentation: no evidence of a wound is present. Wound area is denoted by ▲.

Immediately following fragmentation (0 h) the tissue was disrupted and many cells were sloughing (Figure 2.8). Zooxanthellae, nematocysts, mucocytes, and a few intact epithelial cells could be identified amongst the sloughed cells along with cellular debris adjacent to the exposed tissues. The exposed tissues and structures, including mesoglea, gastrodermis, actinopharynx, and mesenterial filaments contained necrotic cells only near the incision.

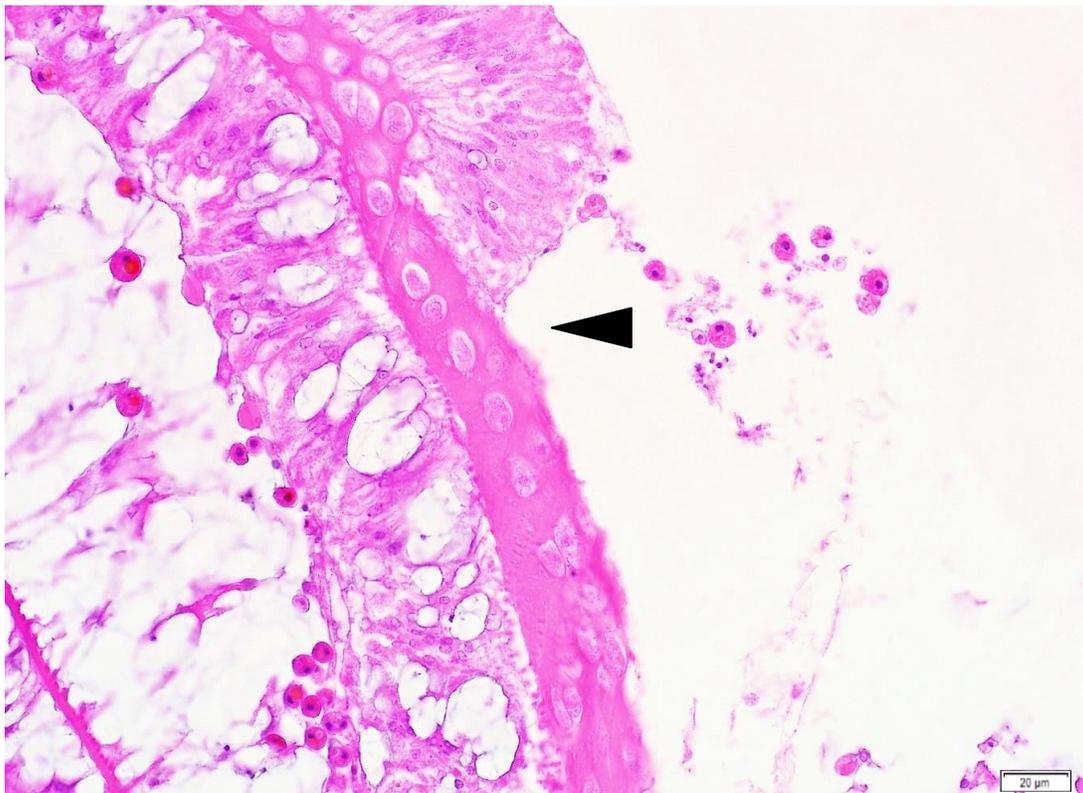


Figure 2.8: *Rhodactis* sp. tissue immediately (0 h) after fragmentation. Disruption of tissue layers resulted in exposure of internal epithelia to the environment and copious sloughing of cells and debris; 400x H&E. Wound denoted by ◄.

At 12 h post-fragmentation the wounded tissues were exposed; however, curling of the external epidermis around the cut surface of mesoglea reduced its exposure (Figure 2.9 A). Mesenterial filaments filled the space between the exposed actinopharynx and the environment, appearing to act as a tissue plug. In one instance mass of free zooxanthellae and nematocysts may have contributed to a tissue plug (Figure 2.9 B); however, this was not a consistent feature at this time. Necrosis was minimal in the tissue closest to the wound, including the epidermis and amoebocytes (Figure 2.9 A).

By 24 hr after fragmentation, some exposed gastrodermis remained but there was little cellular debris adjacent to intact tissues. Mesenterial filaments continued to fill the space between the exposed actinopharynx and the environment. At this time, the leading edge of the regenerating tissue extended inward across the wound area, and this development was composed of undifferentiated cells characterized by a high density of nuclei but there was no evidence of tissue layer separation (Figure 2.10 A - B). The majority of these cells most closely resembled epithelial cells. A notable number of necrotic and/or apoptotic cells was seen in this regenerative margin. Well-differentiated cell types, including cnidocytes and zooxanthellae, were present approximately 120 μm adjacent to the undifferentiated mass. In general, the gastrodermis restored faster than the epidermis. There was unilateral organization as the gastrodermis was intact with mucocytes 140 μm from the surface of the healing margin, while the epidermis was thin above the more well-developed gastrodermis and the columnar structure was lacking (Figure 2.10 A - B). In all instances noted, the undifferentiated cell mass was small (<150 μm) (Figure 2.10 A - B). There was an

increase in the frequency of morphologically altered amoebocytes within the mesoglea closest to the wound, as close as 100 μm to the undifferentiated cell mass in some sections. The cytoplasm of these amoebocytes was indistinct and greatly reduced. The lacunae often appeared to contain apoptotic blebs and/or necrotic debris. (Figure 2.10 C - D). Mitotic figures were not evident.

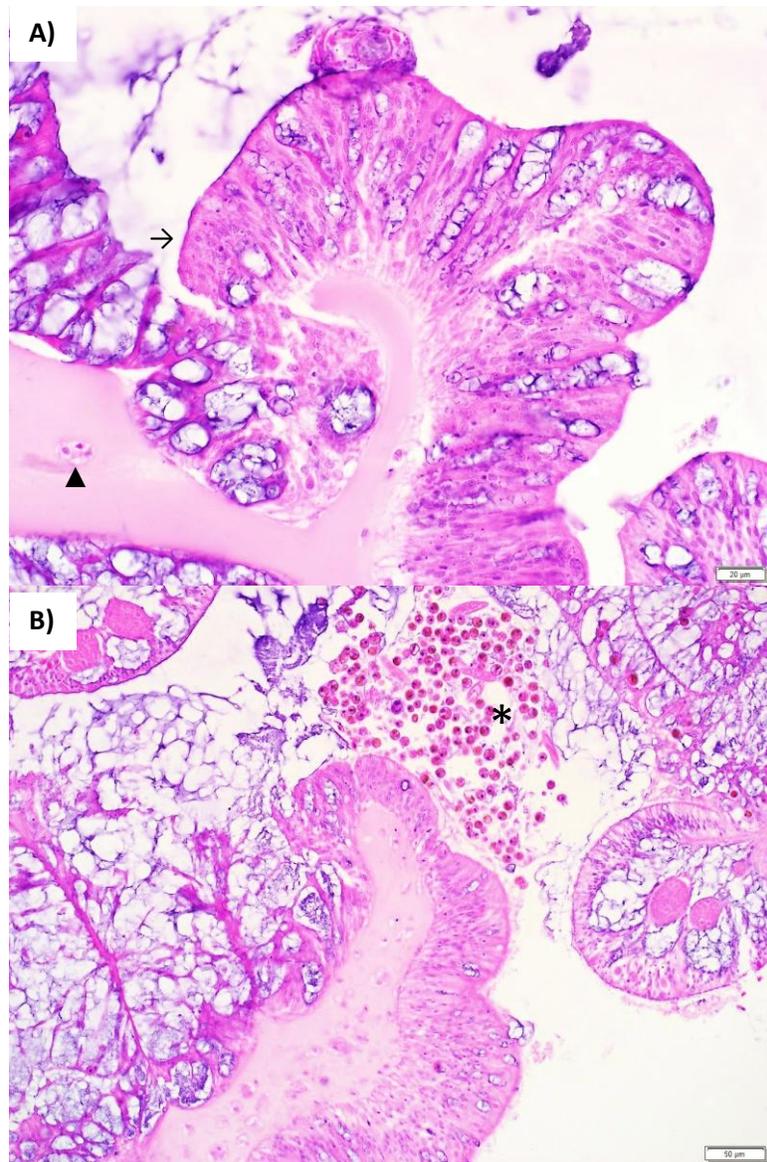


Figure 2.9: *Rhodactis* sp. polyps 12 h post-fragmentation. A) Cut epidermis is curling around mesoglea to reduce exposure to the environment (→). Also note a small number of necrotic cells in the epidermis, as well as the necrotic amoebocytes (▲); 400x H&E. B) Aggregation of free zooxanthellae and nematocysts (*); 200x H&E.

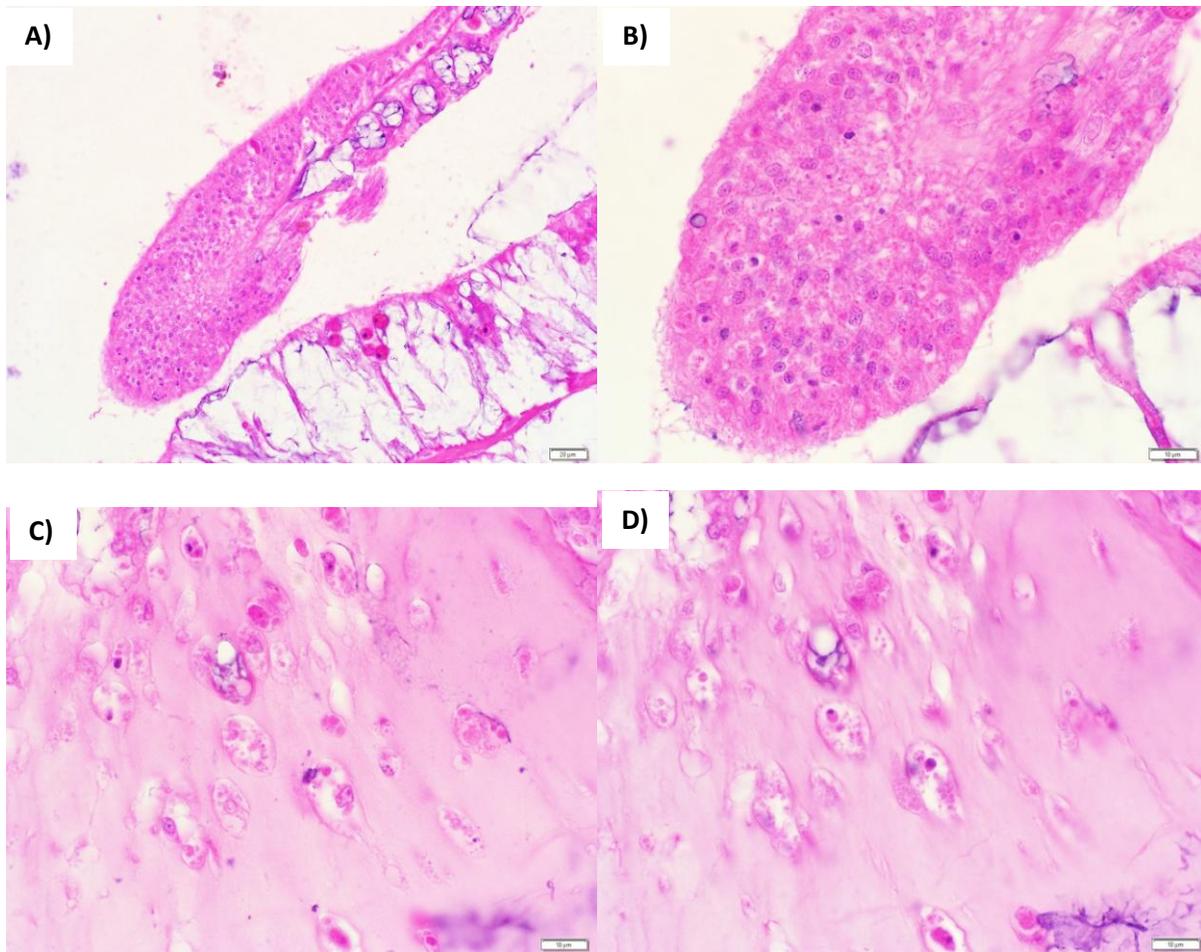


Figure 2.10: Leading regenerative edges of wounded *Rhodactis* sp. tissue 1 d post-fragmentation. A) An undifferentiated mass of cells and debris extended towards the center of the wound. Necrosis was present, and adjacent to the mass all tissue layers were present, with gastrodermis organizing before epidermis; 400x H&E. B) Close up of undifferentiated cell mass; 1000x H&E. C-D) Amoebocytes within the healing margin of the mesoglea. Many amoebocytes within the mesoglea at the healing margin exhibited a reduced and condensed cytoplasm and apoptotic/necrotic nuclei; 1000x H&E.

The tissue patterns noted at 1 d were roughly similar at 2 d after fragmentation. There were similar projections of undifferentiated tissue as well as similar degrees of necrosis/apoptosis in the remodelling tissue. Mesenterial filaments continued to occupy the space between the ruptured actinopharynx and the environment. A particularly notable feature in some polyps at this time was the fusion of the healing epidermis with the actinopharynx epithelium. Reorganization occurred within the epidermis, the epidermis was thick, and mucocytes were present. However, the amount of necrosis and cellular debris present increased in both the area of fusion and also up to approximately 300 μm away. There was still little or no evidence of mitoses (Figure 2.11).

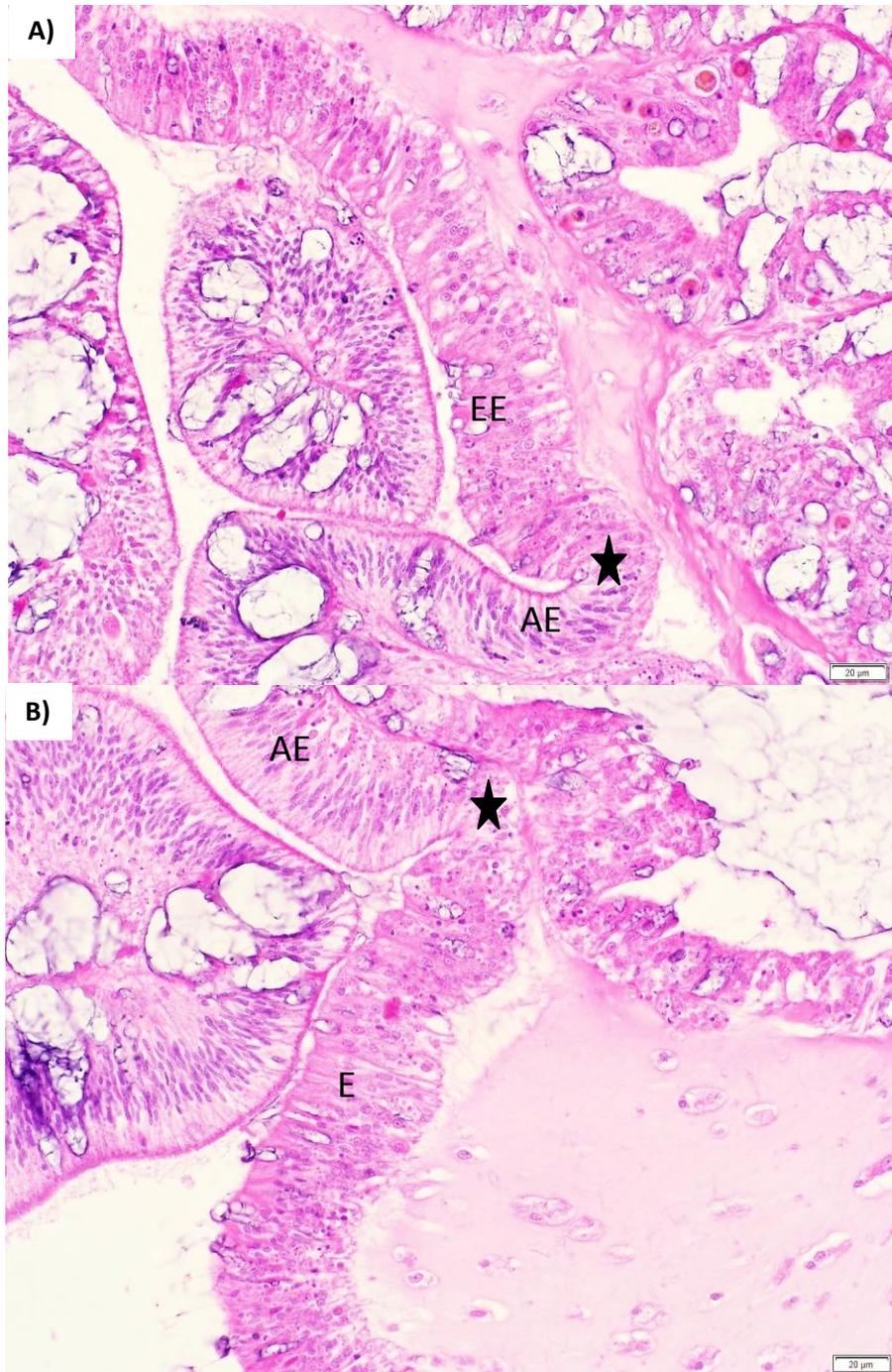


Figure 2.11: Two polyps of *Rhodactis* sp. 2 days post-fragmentation. A-B) The leading edge of the regenerating epidermis (E) has merged and fused (★) with the internal actinopharynx epithelium (AE). Necrosis was severe at this time; 400x H&E.

At 4 d post-fragmentation, a fully-closed wound having continuous surface body wall was found in one individual (Figure 2.12 A) with all three tissue layers present and they were nearly reorganized (Figure 2.12 B). There was copious necrosis in the epidermis and some amoebocytes still exhibited necrotic nuclei with cellular debris present in lacunae (Figure 2.12 B). Mesenteries appeared to be developing by invagination approximately 1000 μm away from the healed site (Figure 2.12 C). As the mesentery developed, complete tissue layers, minimal necrosis, and thinning was found as the tissue reached radially towards the gastrovascular cavity. In another individual that had not yet completed wound closure (Figure 2.13); healthy proximal tissues appeared to be folding over the gap while the epidermal edges of the wound fused with internal actinopharynx epithelium (Figure 2.13 A). Prior to fusion, necrosis was severe, and tissues were atrophied, unorganized (dysplastic), and incompletely differentiated. After the healing edges had merged the number of necrotic cells was reduced and reorganization began (Figure 2.13 B-C).

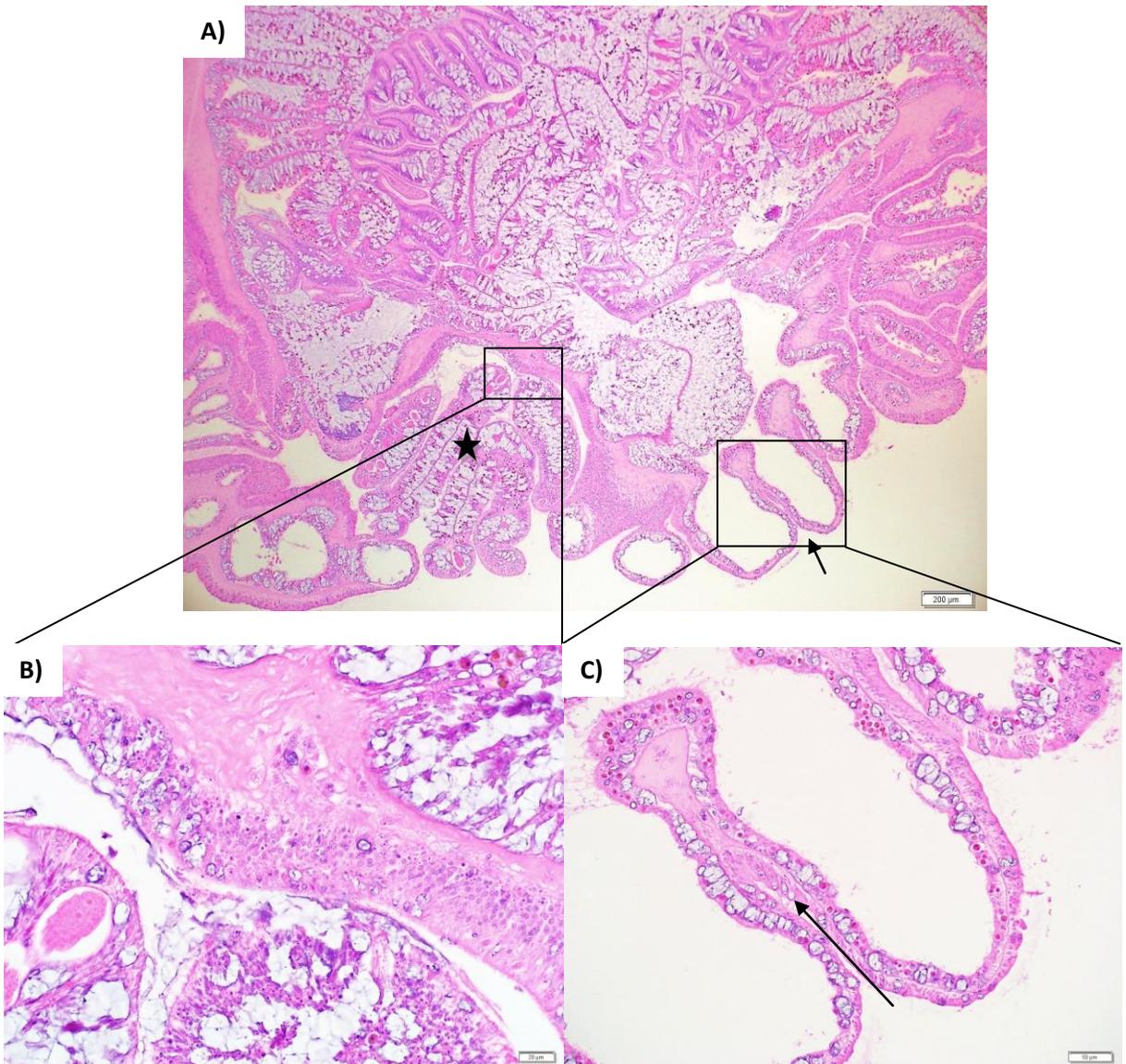


Figure 2.12: Recently healed individual of *Rhodactis* sp. 4 days post-fragmentation. A) Previous wound site (star) and developing mesenteries (arrow); 40x H&E. B) Developing tissue has replaced the wound space. Note the prominent necrosis as the tissue is reorganized; 400x H&E. C) Mesenteries developed by invagination proximal to previous wound site; 200x H&E.

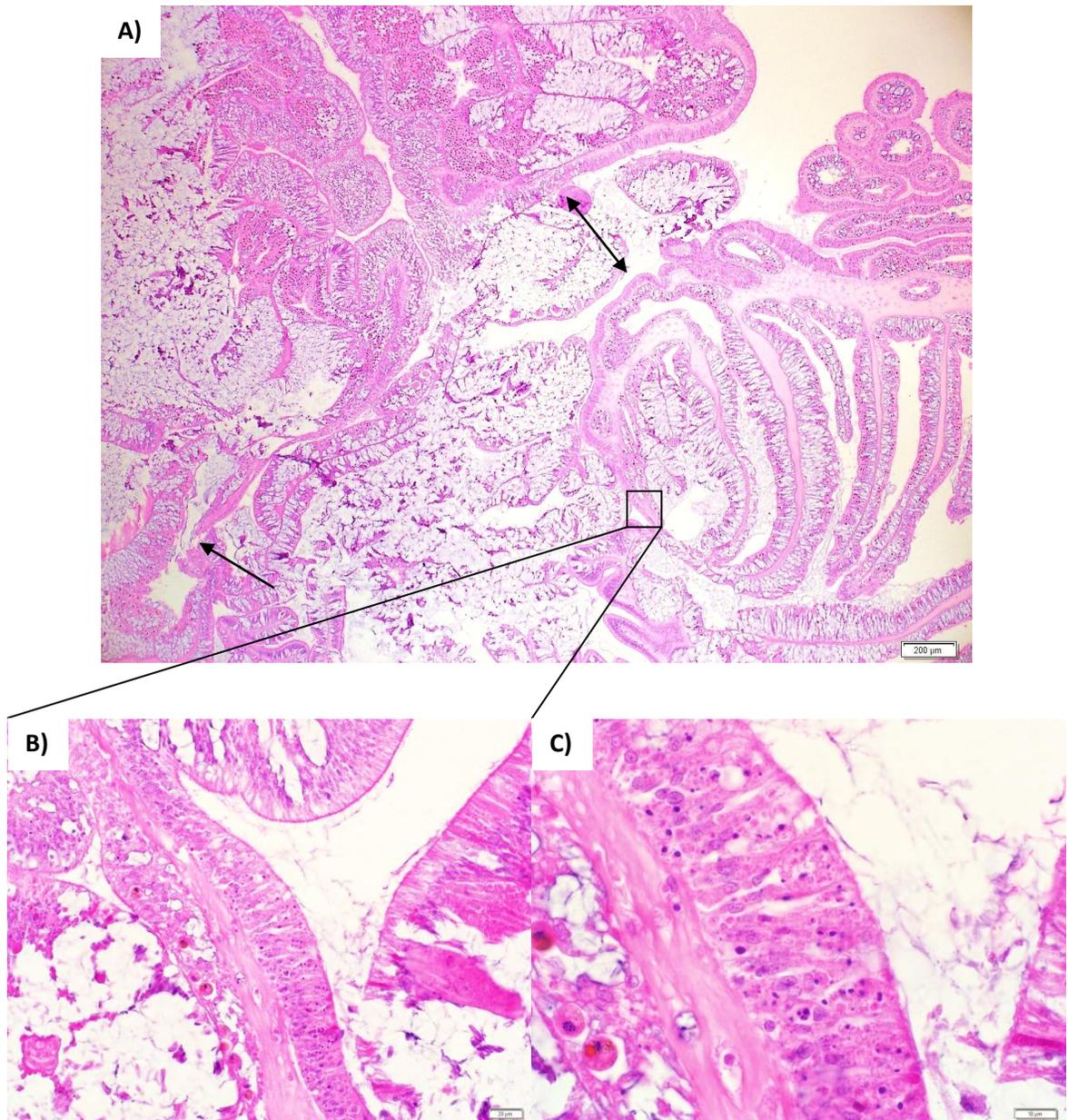


Figure 2.13: *Rhodactis* sp. tissue 4 days post-fragmentation. A) Narrowing of the gap by proximal tissue (double arrow) while healing margin of epidermis fused to actinopharynx (arrow); 40x H&E. B) Complete fusion of epidermis with internal actinopharynx epithelium has occurred; 400x H&E. C) Necrotic cells and debris were very common in the epidermis and the tissue was nearly completely reorganized; 1000x H&E.

By 8 days post-fragmentation 2 of 3 polyps exhibited closed wounds (Figure 2.14 A). In closed wounds, the regenerated tissue exhibited minimal necrosis within the epidermis, none in amoebocytes and the cellular debris noted previously was not present. The epidermis was repopulated by all normally expected cell types, including pigmented granules, mucocytes, and cnidocytes. However, in a polyp that had closed but in which tissue reorganization was relatively delayed, the epidermis was sparsely populated as cells were at low density (Figure 2.14 B). In the one individual that had not fully closed, contraction of the two edges of the wound had occurred bringing the edges within 200 μm from each other (Figure 2.15 A). The fusion of epidermis and actinopharynx epithelia exhibited minimal necrosis compared to what was seen at previously sampled times and all normal cell types were present (Figure 2.15 B). It was apparent in at least one polyp examined at day 8 that the degree of regeneration varied at different levels of sectioning. Regeneration was more advanced in the more ventral sections in which wound closure; fusion of epidermis from either side, was complete. Sections 150 μm dorsal to this demonstrated only fusion of the epidermis to actinopharynx.

Lastly, at 14 d post-fragmentation all wounds were closed and only 1 of 3 polyps exhibited a deficit where the wound has been made. Tissue layers were all present and organized with rare necrosis. In some polyps, epidermis was still not as dense as normal; however, this may have been artifact. The actinopharynx was completely circular again in one instance; however, sectioning of the other two did not catch the full structure of the actinopharynx to say for sure.

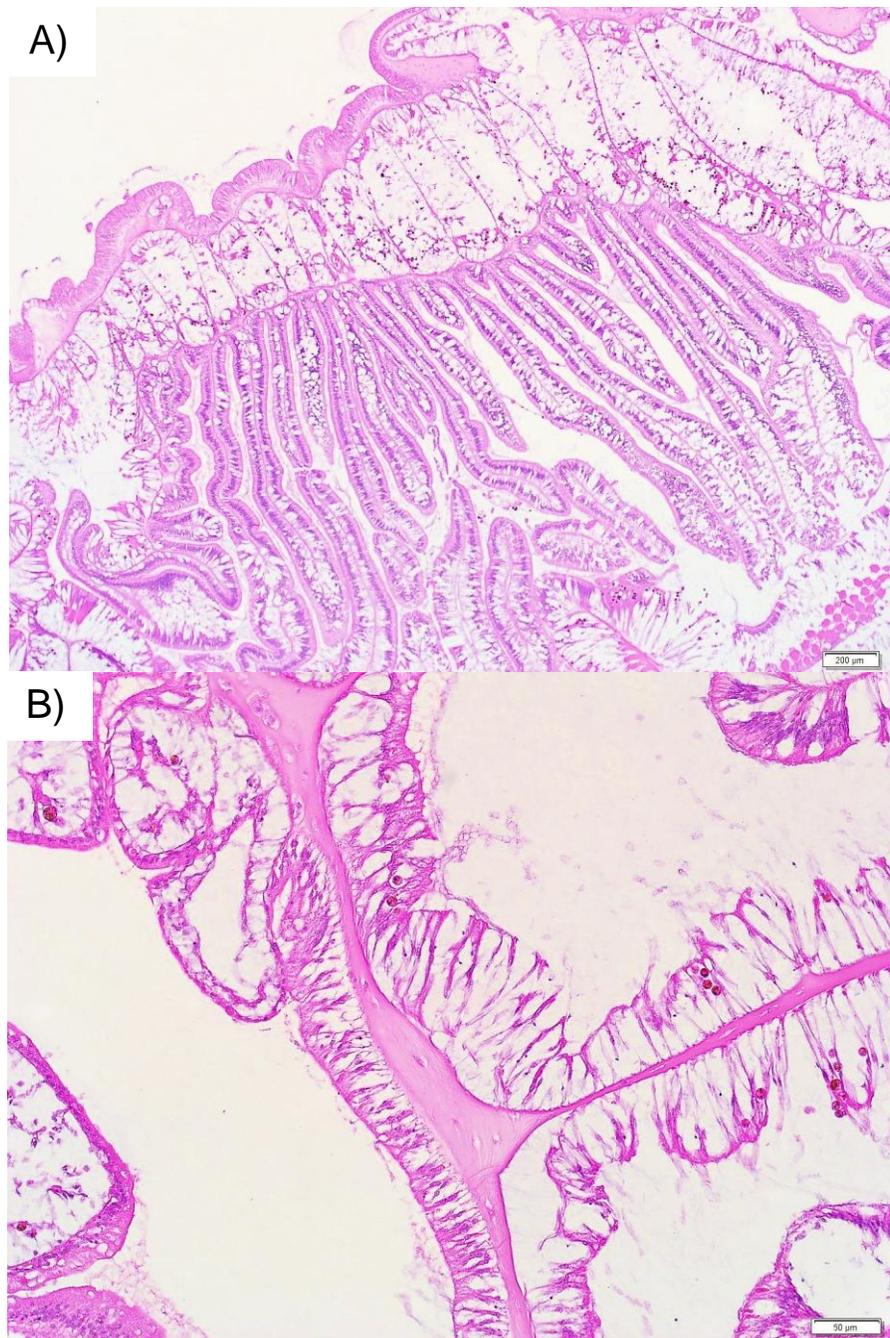


Figure 2.14: *Rhodactis* sp. 8 days post-fragmentation. A) The wound is closed and all tissue layers are all present and organized; 40x H&E. B) The cellular density in new epidermis was greatly reduced; despite this all expected cells types were present and necrosis was absent; 200x H&E.

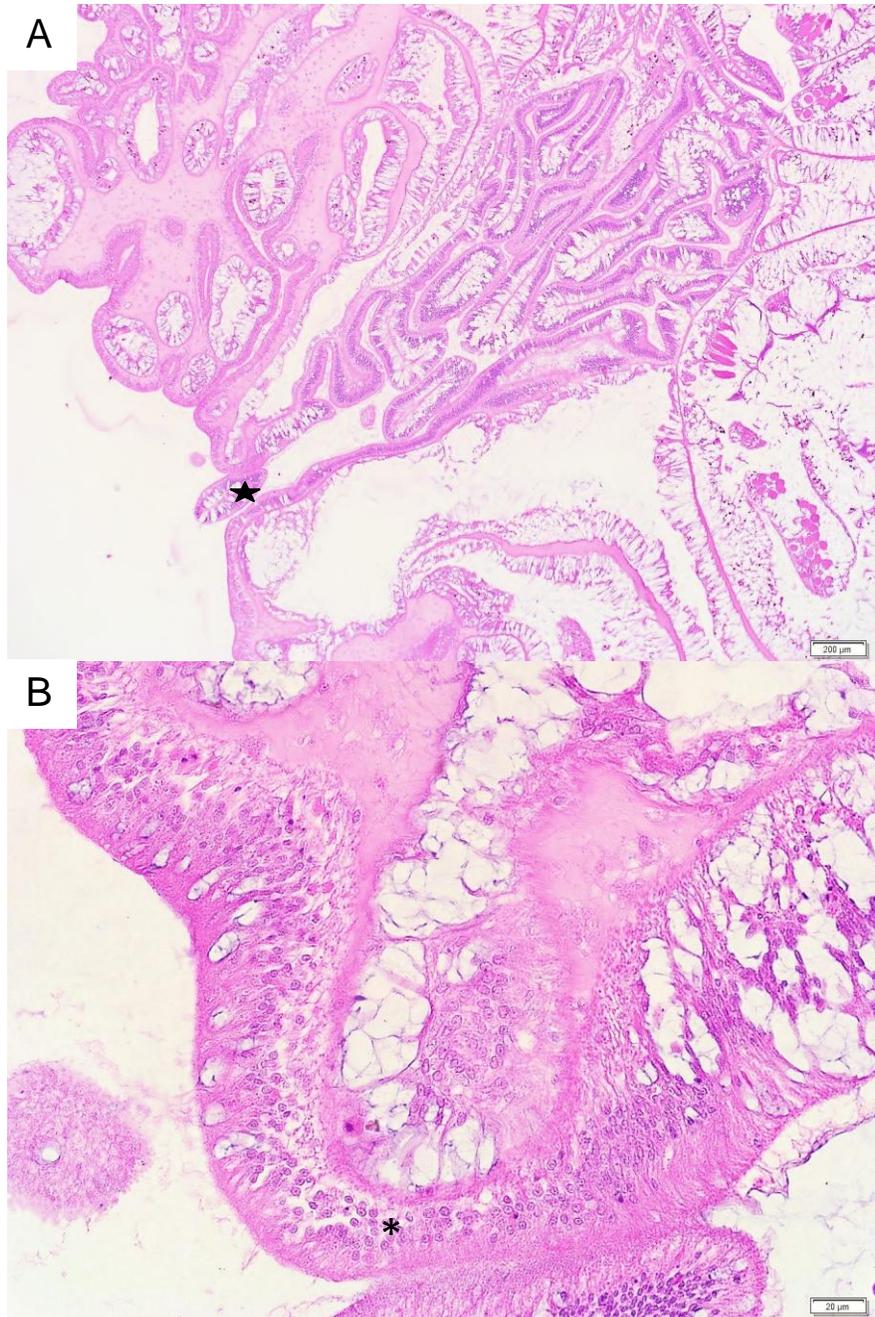


Figure 2.15: *Rhodactis* sp. 8 days post-fragmentation demonstrating incomplete wound closure. A) The gap between the wound (denoted by a star) was greatly reduced; 40x H&E. B) Surface body wall epidermis and actinopharynx body wall epidermis were fused (*) and fully reorganized with rare necrosis/apoptosis; 400x H&E.

2.4.3 Quantitative Results

A significant decrease ($F_{(7,109)} = 8.552$, $p < 0.001$; two-way ANOVA) in square-root-transformed zooxanthellae density occurred at 12 h and 8 d post-fragmentation compared to the control, 1 d, and 2 d samples. Fewer zooxanthellae were present on day 14 than day 1 post fragmentation (Figure 2.16 A). However, no significant differences in zooxanthellae densities were detected among positions 1-3 at any time ($p = 0.060$) (Figure 2.17 A). In contrast, significant differences in amoebocyte density were found over time ($F_{(7,108)} = 4.379$, $p < 0.001$), where density at 0 h was significantly higher than the density at control ($p = 0.001$), 12 h ($p < 0.001$), and 2 d ($p = 0.001$) post fragmentation. Density at 4 d was also significantly higher than at 2 d ($p < 0.001$) (Figure 2.16 B). Amoebocyte density also did not significantly vary among the examined positions over time ($p = 0.997$) (Figure 2.17 B). Epidermal thickness at 4 d was significantly less than at 0 h ($F_{(7,125)} = 1.749$, $p = 0.011$, one-way ANOVA) (Figure 2.16 C). Significant variation in mesoglea thickness occurred over time ($F_{(7, 51.902)} = 3.260$, $p = 0.006$, Welch ANOVA) and the thickness at 14 d was significantly higher than in control individuals (Figure 2.16 D). With both epidermal and mesogelial thickness measurements position and time interactions could not be investigated because assumptions were not met for a two-way ANOVA; however, at position 1 epidermal thinning occurred at 4 d compared to position 2 and 3 (Figure 2.17 C), and mesoglea thickness also was reduced at position 1 at 4 d and 8 d post fragmentation (Figure 2.17 D). Significant decreases in wound width occurred over time ($F_{(5, 4.78)} = 45.352$, $p < 0.001$, Welch ANOVA). Post hoc analysis demonstrated that wound width at 2 ($p = 0.03$), 4

($p=0.005$), 8 ($p=0.013$), and 14 ($p=0.014$) d was significantly lower than at 1 d (Figure 2.18).

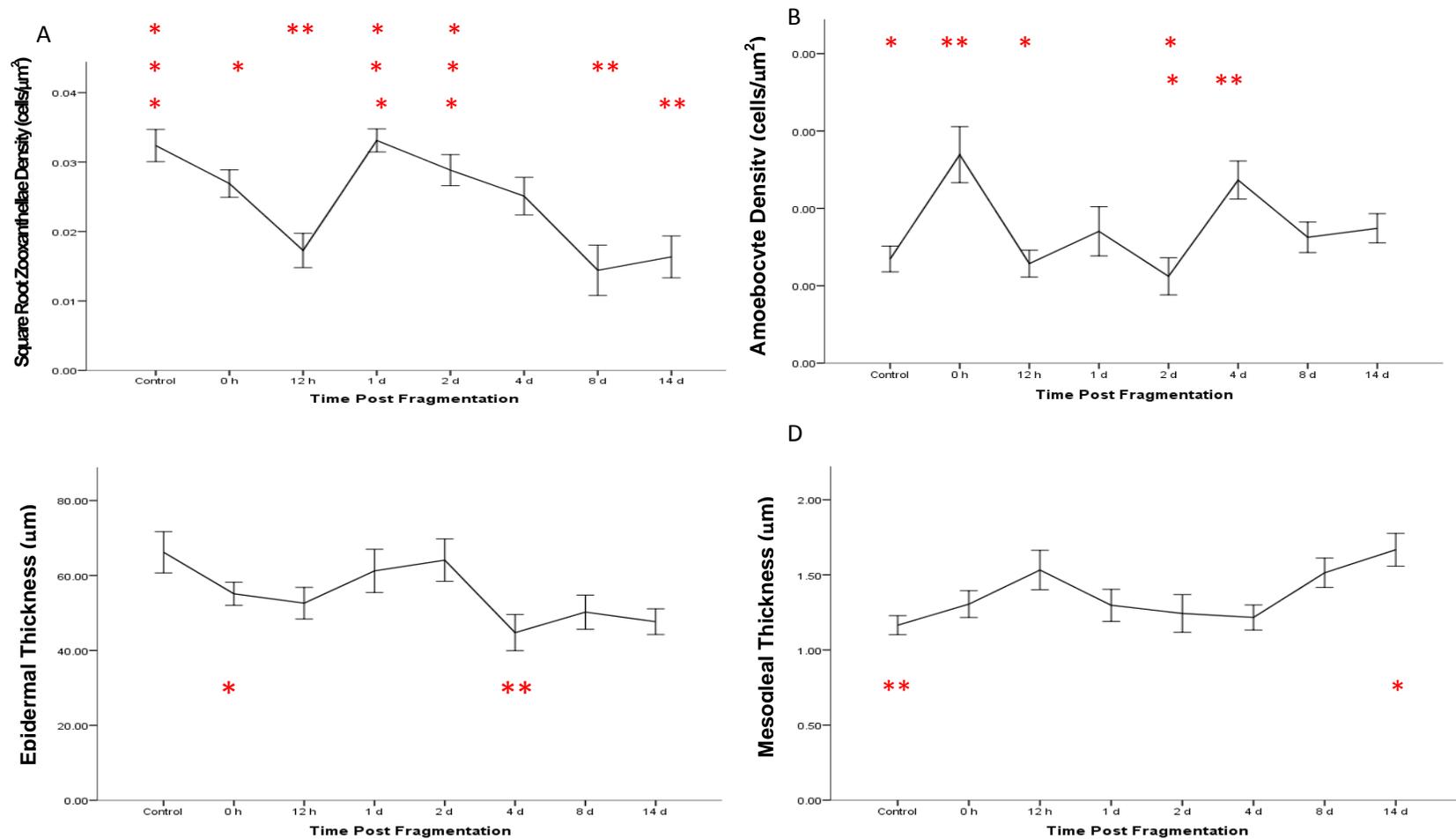


Figure 2.16: Histomorphology of *Rhodactis* sp. following bisection. The mean (\pm SE) of A) zooxanthellae density, B) amoebocyte density, C) epidermal thickness, and D) mesogleal thickness were determined from three individuals at each time point. Groups that do not share the same number of asterix are significantly different ($\alpha=0.05$).

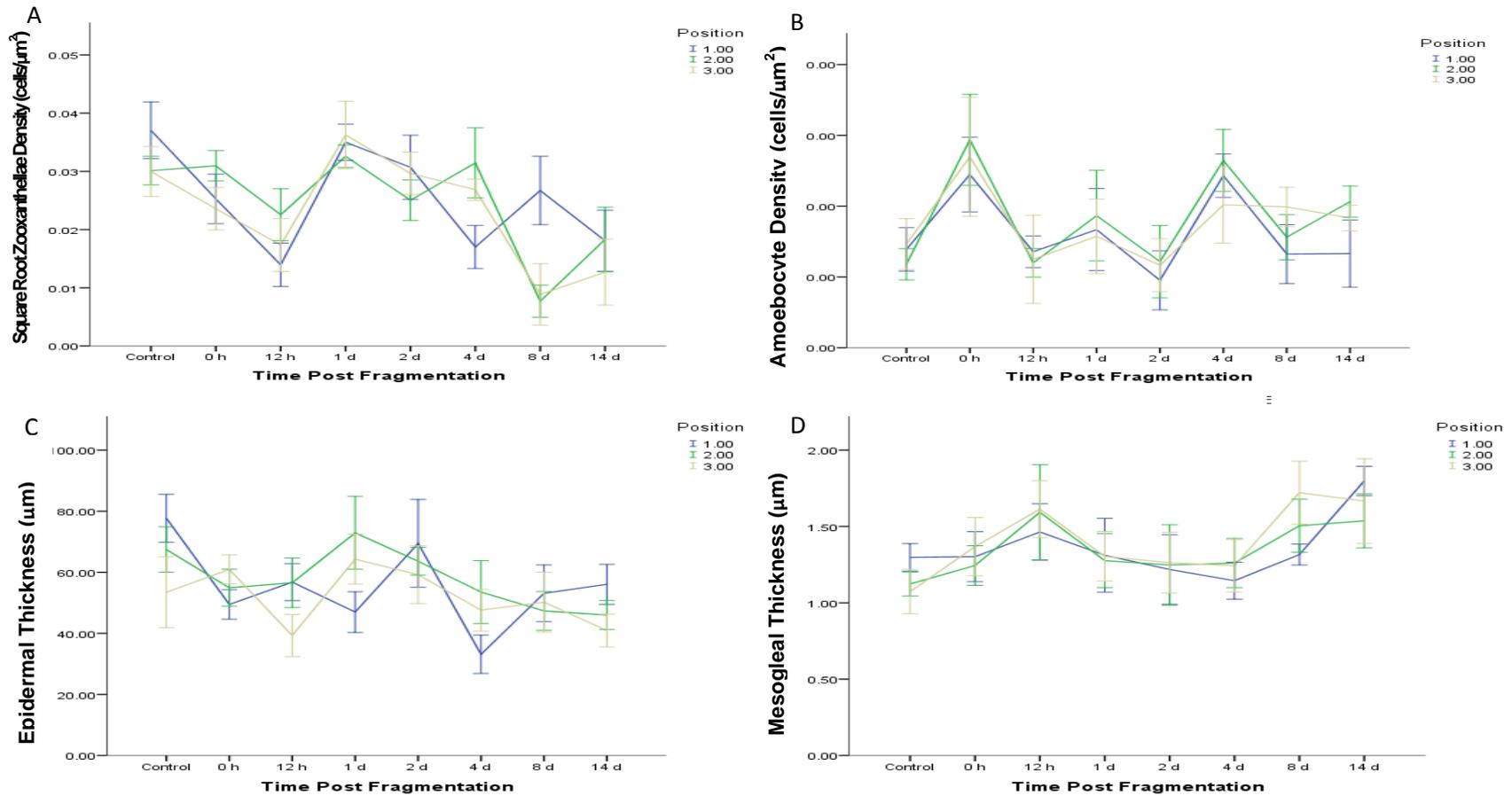


Figure 2.17: Histomorphology of *Rhodactis* sp. following bisection. Position 1 was the measurement closest to the open wound, followed by position 2, and position 3 was furthest from the wound. The mean (\pm SE) of A) zooxanthellae density, B) amoebocyte density, C) epidermal thickness, and D) mesogleal thickness was determined from three individuals at each time point. No significant differences among any positions at any time.

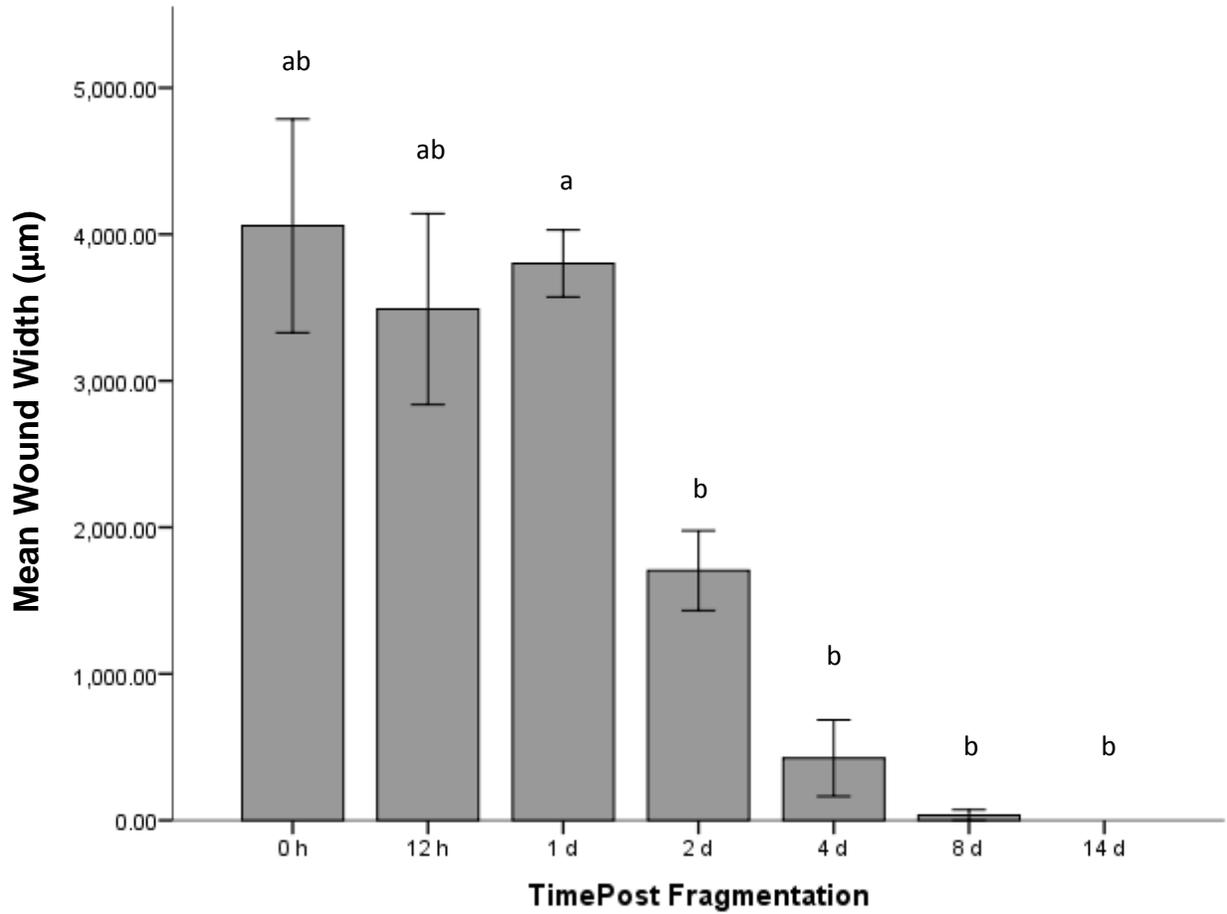


Figure 2.18: Mean (\pm SE) decrease in *Rhodactis* sp. wound width over time after bisection on day 0. Wound width at days 2, 4, 8, and 14 d were significantly lower than at 1 d. Groups that do not share a common letter are significantly different ($\alpha=0.05$). (h=hour, d=day).

2.5 Discussion

Until the present study, very little was known about corallimorpharian regeneration; here I described both the normal histology and the process of regeneration micro- and macroscopically. While some themes were noted during healing of *Rhodactis* sp. that have been described for other corals, there were distinct features that are, to date, unique to this species.

The mucocytes were not PAS positive in the corallimorpharian (Appendix 2.2). Mucocytes and mucus was also found to be PAS-negative in 32 other scleractinian species (Goreau 1956). A few studies on coral histology used other methods to clearly identify mucocytes in the epithelium; including Alcian Blue/PAS (Goldberg 2002; Stolarski et al., 2011), and wheat germ agglutinin staining with fluorescent microscopy (Piggot et al., 2009). PAS is used to stain neutral carbohydrates, whereas Alcian blue stains acidic carbohydrates (Goldberg 2002). Since the mucus is alcian blue-positive, the polysaccharides are suggested to be more acidic in corals (Goldberg 2002). In addition, amoebocytes were PAS-positive in the soft coral *P. fusifera* (Meszaros & Bigger 1999); however, amoebocytes, at least in *Rhodactis* sp., were not PAS positive (Appendix 2.2). Unpublished work on *Gorgonia ventalina* and *G. flabellum* was able to distinctively stain amoebocytes with Luna for eosinophilic granules, PTAH, and Giemsa stains (Esther Peters, personal communication); however, this was not the case with amoebocytes in *Rhodactis* sp. (Appendix 2.2). Further investigation to selectively stain amoebocytes is required to determine the properties of these amoebocytes and to identify whether they infiltrate the wound during regeneration. Lastly, the function of the cells with dense nuclei within the basal portion of the epidermis is unknown. It is

possible that they are examples of single-cell death involved in cell turnover. Staining for apoptosis might help to clarify this. The brown pigmented granules in the epidermis and gastrodermis may be fluorescent pigment granules as seen in many coral species (Labas et al. 2002, Mazel et al. 2003, Oswald et al 2007, Schnitzler et al. 2008); fluorescent microscopy can confirm this.

Quantitative histomorphology demonstrated that wound closure began after 24 h post-fragmentation. Full closure, characterized by a continuous surface body wall, was observed as early as 4 d post-fragmentation and was always completed in the polyps examined at 14 d under the present experimental conditions. The hard coral *M. capitata* had a similar recovery time as tissue was indistinguishable from normal tissue at 12 d post-injury (Work & Aeby 2010). However, *Rhodactis* sp. examined here had recovered grossly and histologically at approximately the same time, while *M. capitata* didn't completely heal and return to normal appearance until 40 d post-injury (Work & Aeby 2010). The discrepancy was mostly due to the lack of pigmentation, or translucency, of the regenerating tissue of hard corals; corallimorpharian re-pigmentation does not lag during regeneration. A lot of coral colouration is known to come from the symbiotic zooxanthellae (Falkowski & Dubinsky 1981; Siebeck et al., 2006). If so in *Rhodactis* sp. zooxanthellae are present within or very near the healing margin and uptake by gastrodermal cells may have continued during regeneration explaining the lack of translucency during regeneration. In addition to zooxanthellae colouration, corals and anemones are known to create green fluorescent-like proteins that provide them with colouration; however, these were not examined in the present study (Lukyanov et al., 2000; Dove et al., 2001; Kelmanson & Matz 2003). Further

research is required to investigate the differences in pigmentation between Corallimorpharia and Scleractinia.

The investigation of specific cell changes determined that zooxanthellae density decreased at 12 h and 8 d post-fragmentation in *Rhodactis* sp. Initially, the symbionts were likely released when the gastrodermis was broken during bisection. However; the zooxanthellae did not aggregate externally as they did in *P. cylindrica* where they contributed to formation of a wound plug (Palmer et al., 2011), nor did they fluctuate in numbers in the adjacent tissue. In the soft coral *P. fusifera* there was an increase in zooxanthellae density in the wound area (Meszaros & Bigger 1999), whereas *P. cylindrica* demonstrated a decrease (Palmer et al., 2011). Zooxanthellae density in wound regeneration may be a species-specific response. In the present study, zooxanthellae density seemed to differ with tissue orientation, and this histomorphology measure was one of the most variable. The 8 d post-fragmentation sections, which had fewest zooxanthellae, also had a gastrodermis that was structurally weak, and zooxanthellae may have been artifactually lost from the sections. The method of fixation should be re-examined to better preserve the gastrodermis. Lastly, because measurements were made only where the gastrodermis was intact, perhaps zooxanthellae were populating the developing tissue to normal density levels as quickly as the gastrodermis was being organized; explaining the lack of significance found.

In the present study, localized amoebocyte density peaked at 0 h and 4 d and both of these peaks were followed by a slight increase in their matrix, the mesoglea. Amoebocytes are known to participate in the inflammatory response in many cnidarians (Patterson & Landolt 1979; Meszaros & Bigger 1999; Olano & Bigger 2000; Mydlarz et

al., 2008; Palmer et al., 2008; Palmer et al., 2011). *P. cylindrica* demonstrated an amoebocyte density increase 6 h post-injury and they aggregated at the exposed edge to seal off tissues (Palmer et al., 2011). The use of amoebocytes to seal exposed tissues was also observed in the soft coral *P. fusifera* 1 d after injury, and by 4 d post-injury there was a notable amoebocyte increase below the seal (Meszaros & Bigger 1999). In these studies the increase in amoebocytes was not within the mesoglea, but rather outside of the mesoglea. The amoebocytes in the *Rhodactis* sp. could not be identified with confidence outside the mesoglea, due to the lack of a differential stain. Amoebocytes had similar nuclei to epithelial cells when stained with H&E. Enumeration of amoebocytes was only possible for these cells within the mesoglea. However, amoebocytes within the mesoglea in *Rhodactis* sp. did undergo a change in morphology during regeneration as early as 12 h post-fragmentation. Amoebocytes have been suggested to have phagocytotic properties in corals (Patterson & Landolt 1979; Hutton & Smith 1996; Olano & Bigger 2000; Mullen et al., 2004; Palmer et al., 2011), and Patterson and Landolt 1978 identified potential lysosomes in altered amoebocytes. Extraction of amoebocytes from *Actina equina* has confirmed phagocytosis in vitro (Hutton & Smith 1999). Extraction of amoebocytes from a corallimorpharian would be useful to determine the contents and properties of the amoebocytes present in *Rhodactis* sp. and potentially to develop immune markers to aid in identification.

The morphology of corallimorpharian regeneration was different than that described for scleractinians. Scleractinia must regenerate tissue above the skeleton if it is present. Corallimorpharians undergo contraction of the whole polyp to bring the cut edges together, unlike scleractinians that cannot readily alter their shape. In *Rhodactis*

sp. the epidermal margins from each side of the wound contained an undifferentiated mass of cells and debris wrapping around the cut mesoglea. This mass was always small with distinct tissue layers visible no more than 120 μm away from the free edge; suggesting that re-organization or remodelling was occurring simultaneously as tissue development progressed. By contracting the oral sphincter muscles, the mass of cells at this cut edge could reach inward towards the actinopharynx where it fused to the actinopharynx epithelia, sealing off the internal tissues from the environment. Then the fused tissues reorganized and mucocytes, etc., differentiated in the epithelia. Necrosis was intense in the epidermis during these stages, and continued to be present during reorganization; however, during this time mitoses were absent in the regenerating tissue when the samples were taken. The tissue at the healing edges then pinched together, bringing the wound edges together and positioning the actinopharynx more centrally. Once closure had occurred, the actinopharynx developed behind the enclosed epidermis to reform a more cylindrical shape, and mesenteries developed by invagination. There was a strong trend of epidermal thinning occurring at 4 d, as the epidermis reached for the actinopharynx. There was individual variation in the timing of this process and factors responsible for this variation could be due to polyp size and condition, or sectioning since some sections didn't capture the undifferentiated tissue mass protecting the bisected edges prior to fusion with the actinopharynx.

M. capitata had re-epithelialized by 8 d post-injury and reorganization had occurred to include expected cell types and numbers (Work & Aeby 2010), which is what we observed in *Rhodactis* sp.. *P. fusifera* re-epithelialized and organized within 3 weeks post-injury (Meszaros & Bigger 1999). *P. cylindrica* was not studied until the end

of regeneration, but at 2 d post-injury there was a developing epithelial front with necrosis of amoebocytes (Palmer et al., 2011). In this study, *Rhodactis* sp. demonstrated a similar unorganized epithelial front with necrosis as early as 1 d post-fragmentation. At 8 d post-fragmentation was the first time sampled that tissues were indistinguishable from healthy tissues.

Other studies put the regeneration process into stages. *P. fusifera* followed the four stages; sealing, repair, fusion, and reorganization (Meszaros & Bigger 1999), and *Porities cylindrica* was described following the four phase pattern of wound healing seen in higher organisms; coagulation, infiltration, proliferation, and remodelling (Palmer et al. 2011). *Rhodactis* sp. regeneration appears to most closely resemble the *P. fusifera* process, but there are similarities and differences with all other studies. Melanin was important in other species in coagulation as the melanin debris from degranulation created a plug, or walled off invaders (Mullen et al., 2004; Mydlarz et al., 2008; Palmer et al., 2011). Corallimorpharians lack melanin in their tissues, but mesenterial filaments have potential to be a plug mechanism as well as a protective measure as they are loaded with nematocysts and were constantly found in the exposed area. Amoebocytes were responsible for the infiltration phase in *P. cylindrica*, and sealing the wound in *P. fusifera*. *Rhodactis* sp. did not experience infiltration that I could tell, and sealing occurred via fusion of surface body wall to the actinopharynx. *M. capitata* was similar to *Rhodactis* sp. in that it didn't elicit an inflammatory response; however, *M. capitata* appeared to lack amoebocytes, unlike *Rhodactis* sp. (Work & Aeby 2010). Proliferation appeared to be absent in *Rhodactis* sp. regenerating tissue because mitotic figures were extremely rare. However, it has been determined in *Acropora cervicornis* that

mitosis occurred mainly at midnight (Gladfelter 1983). Due to daytime sampling in the present study we may have missed any mitoses that occurred. *Rhodactis* sp. regeneration, put simply, occurs by developing extensions of tissue, sealing by fusion to actinopharynx epithelia, followed by reorganization and restructuring.

In conclusion, this study is the first to describe the cellular process of regeneration in any species of Corallimorpharia. We have successfully determined that 14 d is an adequate allotted time for trials studying regeneration and influences of intrinsic and extrinsic factors on regeneration to occur. The process of regeneration in *Rhodactis* sp. demonstrates similarities and differences to other Hexacorallia studies on species in Scleractinia, Actinaria and Alcyonacea. *Rhodactis* sp. does not take part in coagulation by means of cellular debris and degranulation, and the morphological process of fusion between surface body wall and actinopharynx surface body wall has not been seen in other species. More work is required to identify the properties of the undifferentiated mass; whether proliferation is occurring or the migration of cells. Amoebocytes in *Rhodactis* sp. appeared to be different than amoebocytes in other described species and determining a way to selectively stain or extract amoebocytes would be useful to determine the properties and functions of the amoebocytes.

Chapter 3: Intrinsic and Extrinsic Factors That Affect Regeneration in Corallimorpharia

3.1 Abstract

Corals are currently under major stress and in order to understand how intrinsic and extrinsic factors may affect their ability to recovery from injury by means of regeneration, laboratory studies are necessary to isolate parameters. Fragmentation was inflicted and regeneration rate was observed under varying conditions. Lesions consisted of a 50% loss of tissue and regeneration was measured by surface area over time from photos over the course of 14 days. In addition, zooxanthellae densities were measured pre-, mid-, and post-fragmentation with a haemocytometer. Factors studied included polyp size (small $<5 \text{ cm}^2$ vs. large $\geq 5 \text{ cm}^2$), species (*Rhodactis* sp., *Discosoma* sp., and *Ricordea yuma*), light intensity (high $120\text{-}150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ vs. medium $70\text{-}100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ vs. low $30\text{-}60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and food intake (starved vs. fed every 3 days). Polyp size ($p=0.309$) and species ($p=0.275$) did not affect the rate of regeneration. High light ($p=<0.001$) and increased feeding ($p=0.003$) significantly decreased the rate of regeneration from day 2-6. Accompanied with the decreased regeneration rate was an increase of zooxanthellae mid regeneration, although not significant for fed polyps. It is possible that the increased zooxanthellae densities may be diverting energy away from the regenerative process. Overall, my

findings indicate that species and size of polyp did not affect the rate of regeneration, and low light and starvation provided for optimal regeneration rate.

3.2 Introduction

Coral reefs are currently under considerable stress. They are the most biodiverse and productive ecosystems in the world and understanding their ability to recover from a degraded state is imperative. Corals are able to recover in two ways: reproduction, to repopulate an area with new colonies, and regeneration, to recover from current damage to an existing colony. Regeneration is important for reef health as corals are constantly at risk of injury, and failure to regenerate may result in further damage by algal overgrowth or pathogens (Loya 1976; Ward 1995; Meesters et al., 1996; Kramarsky-Winter and Loya 2000; Fine et al. 2002; Pisapia et al 2016). The process of regeneration may be affected by many factors, both intrinsic and extrinsic. Some examples of intrinsic factors that may affect regeneration are reproductive state, colony size, polyp size, morphology, and species. Extrinsic factors include temperature, light, turbidity, sedimentation, and nutrient load.

Generally larger individuals or colonies regenerate faster than smaller ones (Loya 1976, Smith and Hughes 1999, Kramarsky-Winter and Loya 2000, Oren et al. 2001, Soon and Chen 2003, Henry and Hart 2005). In *Acropora pulchra*, a branching coral, longer fragments (4-7 cm) had increased skeletal extension rates compared to small fragments (1 cm) (Soong and Chen 2003). In *Fungia granulosa*, a single polyp species, large individuals regenerated faster than small individuals, and small individuals often died (Kramarsky-Winter and Loya 2000). The death of small corals

after injury has been documented in many studies (Loya 1976, Ward 1995, Meesters et al 1996, Smith and Hughes 1999, Kramarsky-Winter and Loya 2000) suggesting that larger colonies are less susceptible to any detrimental effects. Regeneration rate has also been seen to vary between species in the same location (Bak and Steward-Van Es 1980, Wahle 1983, Hall 1997). The difference tends to be due to morphology of the coral (Hall 1997, Smith and Hughes 1999). Branching coral species regenerate much faster than massive and submassive coral species (Hall 1997). However, morphologically similar species can also vary in regeneration rate; demonstrated by plexaurid soft corals in both shallow and deep zones on the reef *Eunicea mammosa* regenerated the fastest, followed by *P. homomalla* then *P. dichotoma* (Whale 1983). An individuals' reproductive state can also affect the rate of regeneration. *Fungia granulosa* polyps that were undergoing gametogenesis were not able to completely regenerate regardless of individual or lesion size (Kramarsky-Winter and Loya 2000). It's important to understand the effect of intrinsic factors on regeneration rates before studies on extrinsic factors can take place, so that they may be controlled for.

Extrinsic factors have been studied in the past for their impact on coral biological function. Light intensity correlates with depth, and corals in shallow environments tend to have increased growth and/or regeneration (Nagelkerken et al., 1999; Soong & Chen 2003; Fisher et al., 2007; Denis et al., 2013). As photosynthetically active radiation, or intensity of light, increases, corallimorpharians increase the densities of zooxanthellae in tissues (Kuguru et al., 2007). Zooxanthellae are an important energy source for the host that can allow for greater growth (Wooldrige 2010). However, there may be an upper limit of zooxanthellae density that when breached can cause negative effects on

the host (Wooldrige 2010). This has been observed in scleractinians, but not in any corallimorpharians to date. Nutrients are another extrinsic factor that effect growth and regeneration. Nutrients in the form of ammonia from fish communities can increase both growth and regeneration rate (Porat & Chadwick-Furman 2005). Additionally, in *Pocillopora damicornis* nitrogen enriched waters caused a doubling of zooxanthellae densities within the host (Stimson & Kinzie 1991). However, the high levels of zooxanthellae had a negative effect on the *P. damicornis* physiology as less photosynthates were translocated to the host. Extrinsic factors often have optimal ranges, and identifying the optimal ranges will be useful in further laboratory studies.

Many studies focusing on extrinsic factors and coral regeneration are carried out in the field where it can be difficult to isolate confounding parameters. In the fall, when average water temperatures are higher (26°C) regeneration was faster than in *F. granulosa* during the spring (21°C) (Kramarsky-Winter and Loya 2000). A complementary laboratory study using the same species tested both temperatures during each season, and determined that the effect was due to a combination of both temperature and reproductive state, rather than due to temperature alone (Kramarsky-Winter and Loya 2000). Laboratory studies can be very useful for identifying the effects of different parameters.

Corallimorpharia are Anthozoa that are closely related to Scleractina, the reef building corals; however, they lack a calcareous skeleton. Corallimorpharia are easily maintained and repair in a matter of weeks as opposed to months for Scleractinia (Loya 1976, Nagelkerken et al. 1999, Kramarsky-Winter and Loya 2000, Oren et al. 2001, Denis et al. 2013). Tropical species within the order Corallimorpharia are popular in the

aquarium trade for their ease of culture and propagation via fragmentation (Sprung 2002). Given the challenges of isolating experimental parameters in field studies, and a need for laboratory studies, Corallimorpharia are an ideal candidate for a model laboratory species to study many aspects of coral biology.

The purpose of this study was to therefore to determine the effects of intrinsic and extrinsic factors on regeneration in Corallimorpharia. The intrinsic factors studied were species and size, while extrinsic factors examined included light and feeding. It is hypothesized that 1) given different species prefer different depths along the reef gradient, those that naturally prefer shallow areas will regenerate faster than those that prefer deeper depths. 2) Given some cnidarians regenerate by morphogenesis, smaller individuals have reduced cellularity and zooxanthellae, reducing their ability to regenerate quickly. 3) Increased light intensity allows for increased zooxanthellae density which provides energy for faster regeneration. 4) Regeneration is dependent, in part, on nutrient intake, so feeding will increase the rate of regeneration. Polyps were fragmented into equal halves in the laboratory and regeneration rate was measured by polyp surface area over time. Zooxanthellae density during regeneration was also measured to attempt to explain any differences in regeneration rate.

3.3 Materials and Methods

3.3.1 General Conditions

Water parameters were measured three times per week; twice using a Master kit (Aquarium Pharmaceuticals, Chalfont PA USA) and once with a HACH DR2800 Spectrophotometer (HACH Company, Loveland, Co USA). Ammonia, nitrite, and nitrate

were maintained at appropriate levels of 0-0.1, 0 and 0-15 ppm, respectively. pH was maintained between 8.2-8.3 (Accumet AB15 Plus pH Meter; Thermo Fisher Scientific Inc, Ottawa ON CA) with a biweekly addition of reef buffer (Seachem, Madison GA USA) and 10% water changes were carried out once per week. A 20 cm diameter protein skimmer (Hagen Aqualab, Guelph ON CA) as well as a red mangrove (*Rhizophora mangle*) refugium of 50 pods were used to maintain water quality. The seawater was prepared with synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH USA) dissolved in deionized water to achieve a salinity of 34 ppt (+/- 1 ppt). Salinity was checked daily, and water lost to evaporation was replenished with deionized water. A titanium plate heat exchanger (Armstrong Fluid Technology, Toronto ON CA) maintained a temperature of 25 °C ($\pm 1^{\circ}\text{C}$).

Corals were housed under Radion G3 LED lights (Ecotech Marine, Allentown PA USA) on a 10:14 hr light:dark cycle. Lights were programmed based on hobby standards to emit 100% UV, royal blue, and blue, 15% white and green, and 20% red, all at 60% brightness (Ecosmart Live, Ecotech Marine). Feeding opportunities occurred once per week using Ultra MinF fine food (Fauna Marin, Germany) saturated in Ultra MinD amino acid supplement (Fauna Marin, Germany). Water flow was turned off for 30 min, and the food suspension was pipetted over the surface of each polyp.

Three species within the order Corallimorpharia were imported from Java Indonesia from the aquarium trade (Reef Wholesale, Toronto ON). The three species represented three genera, including *Discosoma*, *Rhodactis*, and *Ricordea*. Colonies were removed from their rock substrate after a minimum of three days in quarantine using a combination of scalpels and bone cutters to break up the rock below the polyps.

Corals were then allowed a minimum of one week to heal on 4.5 cm diameter round ceramic discs in 20.8 L experimental tanks that were 40.6 x 20.3 x 25.4 cm in dimension and that were on the same recirculating system as the holding tanks.

3.3.2 *Experimental Design*

A total of four factors were tested in two experimental trials, and then a replicate trial was carried out for three of the factors (species excluded). The first trial focused on the effect of the intrinsic factors: species and initial polyp size on regeneration rate. Three species (*Rhodactis* sp., *Discosoma* sp., and *Ricordea yuma*) as well as two different size classes of each species, small (surface area $<5 \text{ cm}^2$) and large (surface area $\geq 5 \text{ cm}^2$), were examined. The day prior to fragmentation surface area measurements were taken from photographs (Image J; Abramoff et al. 2004) to divide polyps into the two size classes. In the second trial, extrinsic factors including lighting and feeding were examined. There were three lighting regimes; high (100% brightness), medium (60% brightness), and low (20% brightness), as well as two feeding regimes; starved and high feeding (fed every 3 days). In the replicate trial only high and low light regimes were used. Again, the day prior to fragmentation surface area measurements from photographs using Image J were taken (Abramoff et al. 2004), this time to ensure only polyps $\geq 5 \text{ cm}^2$ were used in case size variation (from the intrinsic study) caused differences in regeneration rates.

Each treatment combination group consisted of 8 whole polyps, which were then fragmented into equal halves across the mouth using a scalpel creating 50% tissue loss. Using a random number generator (www.random.org), polyps were assigned to a

given treatment as well as a random placement in an aquarium. Light brightness was then altered in the extrinsic study; in the high light tanks brightness was increased to 100% while other parameters remained the same resulting in 120-150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photosynthetically active radiations (PAR) (MQ-200 Quantum Light PAR Meter, Apogee Instruments, Logan UT, USA). Medium lighted tanks were exposed to the same brightness of the maintenance conditions (60%) resulting in 70-100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Low light treatment tanks had a brightness that was decreased to 20% to emit 30-60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

3.3.3 Sampling

3.3.3.1 Regeneration Rate

Using the same random number generator, 4 - 8 50% fragments from each treatment were chosen to remain in place to obtain regeneration rate measurements. Regeneration rate was measured as the change in surface area (cm^2) over time from photos (ImageJ) taken of chosen polyps using a ruler for scale on days 2, 6, 10 and 14 at 1400 h each day. Change in surface area (cm^2) for early (day 2 - day 6), mid (day 6 - 10), and late (day 10 - 14) phases were calculated.

3.3.2 Zooxanthellae Density

Four randomly chosen fragments per treatment were sampled for zooxanthellae density measurements. Samples from each treatment were taken on day 0 (pre-fragmentation), 7 (mid-regeneration) and 14 (end of regeneration) by cutting a wedge (~5mm at the thickest edge) from a site distant from the injury. This wedge was placed into 1 mL of filtered seawater (FSW) (0.2 μm ; Rapid Flow Filter with aPES membrane,

Fisher Scientific, Ottawa ON, CA) then placed on ice for short transportation. In the laboratory, zooxanthellae was isolated from tissue and zooxanthellae density measurements were made using an Improved Neubauer Hemocytometer (Hausser Scientific, Horsham, PA USA). Briefly, fragments were weighed to 0.0001 g, placed into a Waring blender (Waring Laboratory Science, Winsted, CT USA) with 15 mL FSW and homogenized for 2 min. Samples were then centrifuged at 2800 g for 5 min. The supernatant was discarded, and the pellet was washed in 10 mL FSW and recentrifuged. The pellet was resuspended in 10 mL FSW and zooxanthellae were enumerated in the hemocytometer. Each sample was counted four times to obtain a mean zooxanthellae count expressed as cells per μL , which was then expressed as cells per g wet weight, as follows.

$$\text{Zooxanthellae density 1} \left(\frac{\text{cells}}{\mu\text{L}} \right) = \frac{\text{average cells counted per square} * \text{dilution factor}}{\text{volume of a square} (\mu\text{L})}$$

$$\text{Zooxanthellae density 2} \left(\frac{\text{cells}}{\text{mL}} \right) = \text{Zooxanthellae density 1} \left(\frac{\text{cells}}{\mu\text{L}} \right) * 1000$$

$$\text{Zooxanthellae density} \left(\frac{\text{cells}}{\text{g wet weight}} \right) = \frac{\text{Zooxanthellae density 2} \left(\frac{\text{cells}}{\text{mL}} \right)}{\left(\frac{1}{\text{wet weight}} \right)}$$

3.3.4 Statistical Analysis

In SPSS (IMB Statistical Software) outliers were identified and removed, normality examined with use of a Shapiro-Wilk test ($\alpha=0.05$), and transformations were performed if $\alpha<0.05$. Trial differences, between original and replicate, were tested and data were combined if there were no significant differences. Levene's test validated

equal variances for two way ANOVAs, and Mauchly's test validated, or allowed corrections for sphericity for repeated measures ANOVAs. For regeneration rates a two-way repeated measures ANOVA was used to examine the effect of factors (species, size, light, and feeding), phase, and interactions on changes in surface area (cm²). The ANOVA for intrinsic factors, species and size had surface area at day 2 as a covariate to account for the initial size differences. A two-way ANOVA was conducted to examine the effect of species, size, lighting, feeding and phase on zooxanthellae density (cells/g wet weight) during regeneration. Again normality examined using a Shapiro-Wilk test ($\alpha=0.05$) and data were transformed if $\alpha<0.05$. When data violated the equal variance assumption; separate one-way ANOVAs were conducted.

3.4 Results

3.4.1 Regeneration Rate

A repeated measures ANOVA examined the effect of size (large and small) and species (*Discosoma* sp., *Rhodactis* sp., and *Ricordea yuma*) on regeneration rate at three different time periods (early, mid and late phases) during the first intrinsic trial. Using surface area at day 2 as a covariate to account for the size differences between the two size classes, there was no significant effect of phase ($p=0.050$), nor the interaction with phase and intrinsic factors, size ($p=0.359$, Figure 3.1 A), or species ($p=0.298$, Figure 3.1 B), on regeneration rate. However, when no covariate was used, phase alone demonstrated a significantly greater increase in surface area in the early phase (1.079 ± 0.269 cm²) compared to the mid (0.003 ± 0.145 cm², $p=0.024$) and late phases (0.037 ± 0.222 cm², $p=0.007$) (Figure 3.2). Repeated measures ANOVA for the

repeat trial confirmed no significant effects of phase or species on regeneration rate, and lack of covariate did not demonstrate any differences in phases that were seen in the original trial.

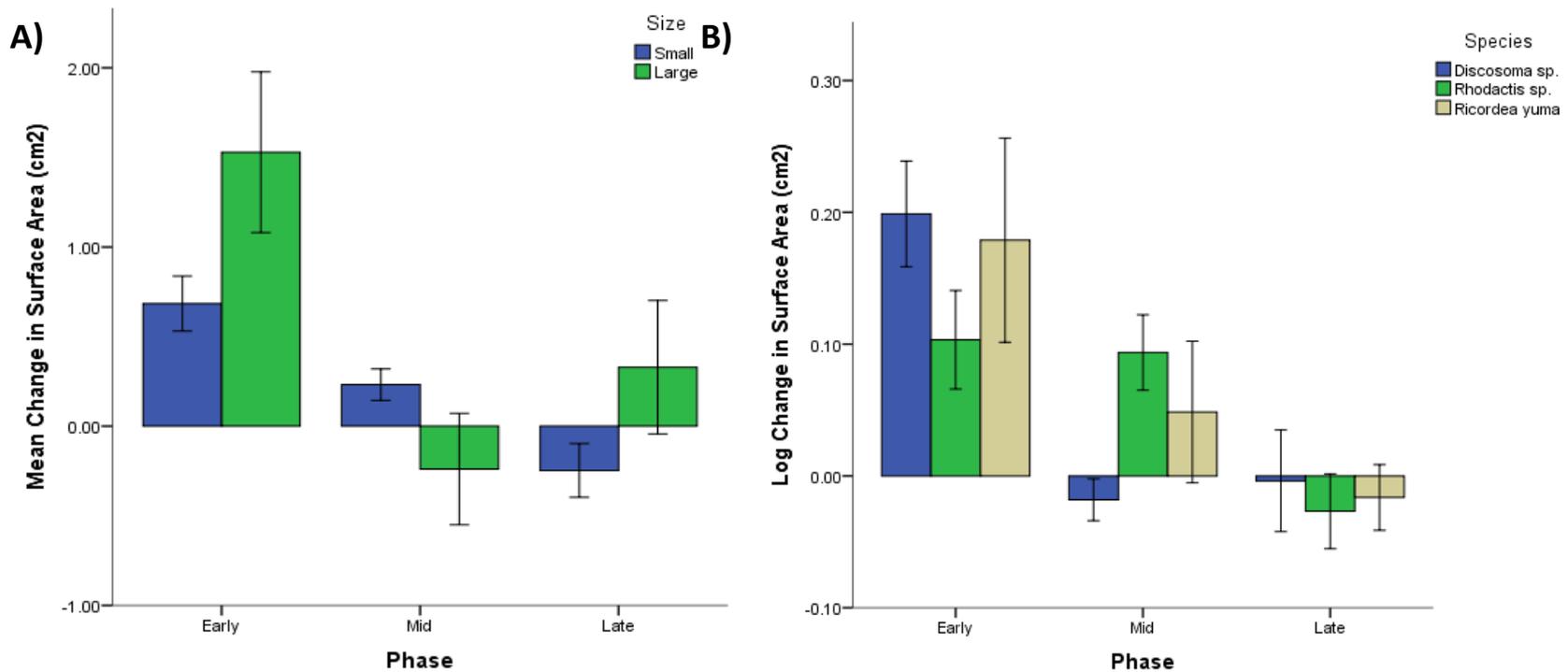


Figure 3.1: Mean (\pm SE) change in surface area among three species of corallimorpharians at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. A) No significant difference in size was observed between large (≥ 5 cm² surface area) and small (< 5 cm² surface area) polyps (n=10 per group). B) There were no significant differences in the ability of three different species of Corallimorpharia to regenerate after fragmentation (n=6-8 per group).

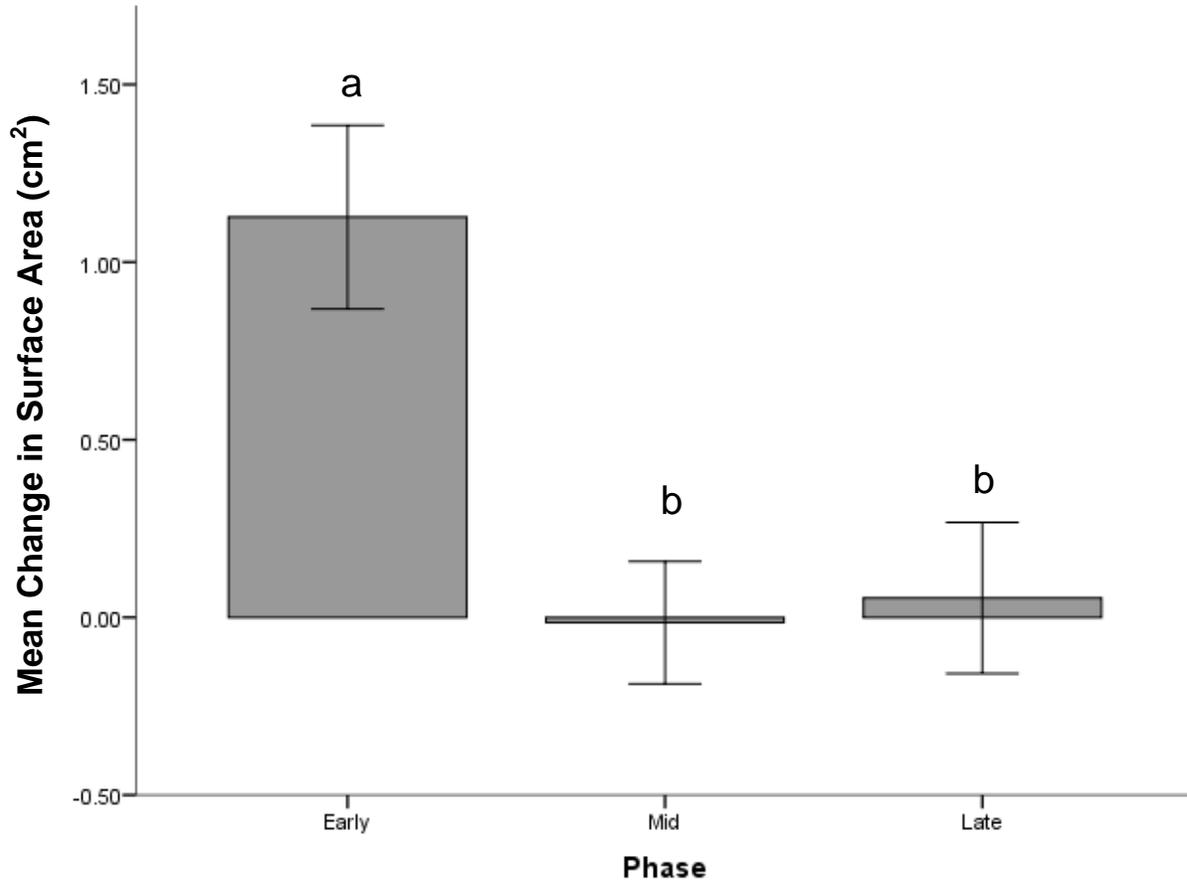


Figure 3.2: Mean (\pm SE) change in surface area among three species of corallimorpharians at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. Polyps regenerated significantly more tissue in the early phase than in the mid ($p=0.024$) and late phases ($p=0.007$) ($n=23$ per group).

The second trial on extrinsic factors, original trial data and repeat trial data were combined (two-way repeated measures ANOVA, using log transformed data and a Huynh-Feldt degrees of freedom correction for extrinsic factors) and there was a significant main effect of phase on change in surface area ($F_{(1.999, 118)}=7.133, p=0.001$), as well as significant interaction between phase and light ($F_{(3.997, 118)}=6.136, p<0.001$), and phase and feeding ($F_{(1.999, 118)}=6.276, p=0.003$). Polyps regenerated significantly less tissue during the late phase ($0.041 \pm 0.007 \log \text{cm}^2$) (day 10-14) of regeneration than in the early ($0.103 \pm 0.014 \log \text{cm}^2, p=0.001$) and mid ($0.078 \pm 0.011 \log \text{cm}^2, p=0.033$) phases.

The impact of light on regeneration was substantial. Polyps regenerated markedly less tissue during the early phase ($0.014 \pm 0.023 \log \text{cm}^2$) when held in high light conditions compared to those exposed to medium ($0.121 \pm 0.029 \log \text{cm}^2, p=0.016$) and low light ($0.175 \pm 0.022 \log \text{cm}^2, p<0.001$) (Figure 3.3). Those polyps held in high light regenerated significantly more during the mid ($0.086 \pm 0.018 \log \text{cm}^2$) than the late phase ($0.022 \pm 0.012 \log \text{cm}^2, p=0.020$). Low light treated polyps regenerated the most tissue during the early phase ($0.175 \pm 0.022 \log \text{cm}^2$) compared to mid ($0.074 \pm 0.017 \log \text{cm}^2, p=0.003$) and late phases ($0.050 \pm 0.011 \log \text{cm}^2, p<0.001$).

Polyps that were not fed regenerated significantly more tissue ($0.149 \pm 0.020 \log \text{cm}^2$) during the early phase than those polyps that were given food ($0.058 \pm 0.021 \log \text{cm}^2, p=0.003$) (Figure 3.4). In addition, starved polyps regenerated significantly more tissue in the early phase than in either of the mid ($0.065 \pm 0.016 \log \text{cm}^2, p=0.009$) or late phases ($0.045 \pm 0.010 \log \text{cm}^2, p<0.001$). In contrast, polyps that were fed regenerated significantly more tissue during the mid ($0.090 \pm 0.016 \log \text{cm}^2$) than the

late phase ($0.036 \pm 0.010 \log \text{ cm}^2$, $p=0.027$), but not more than the early phase ($0.058 \pm 0.021 \log \text{ cm}^2$, $p=1.00$).

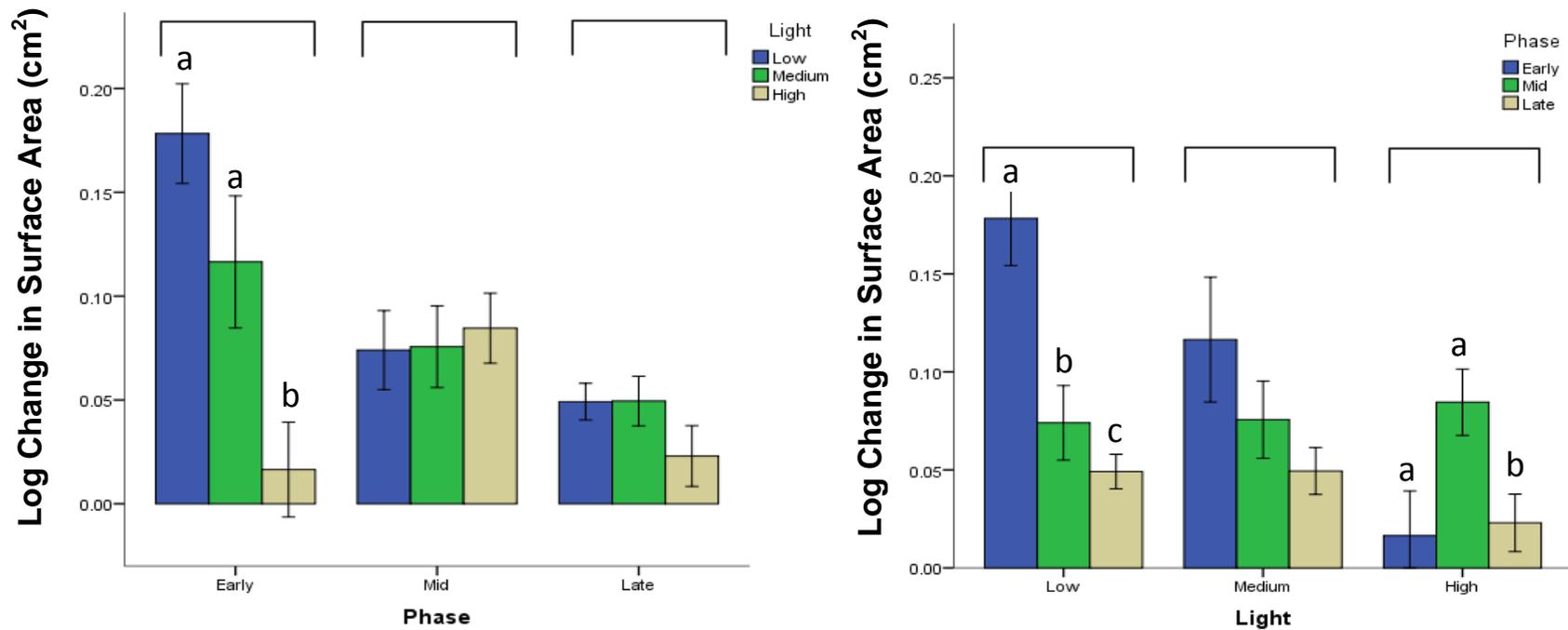


Figure 3.3: Mean (\pm SE) log change in surface area of *Rhodactis* sp. individuals at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation under different light treatments (high = 120-150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, medium = 70-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and low = 30-60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A) In the early phase, polyps in high light regenerated significantly less tissue compared to those in medium ($p=0.016$) and low light ($p<0.001$). Individuals in mid and late phases of regeneration were not affected by light treatment. B) Among light treatments, polyps under low light demonstrated a significant decline in regeneration rate over time, and under high light exhibited a peak in regeneration in the mid phase of regeneration ($n=15-26$ per treatment).

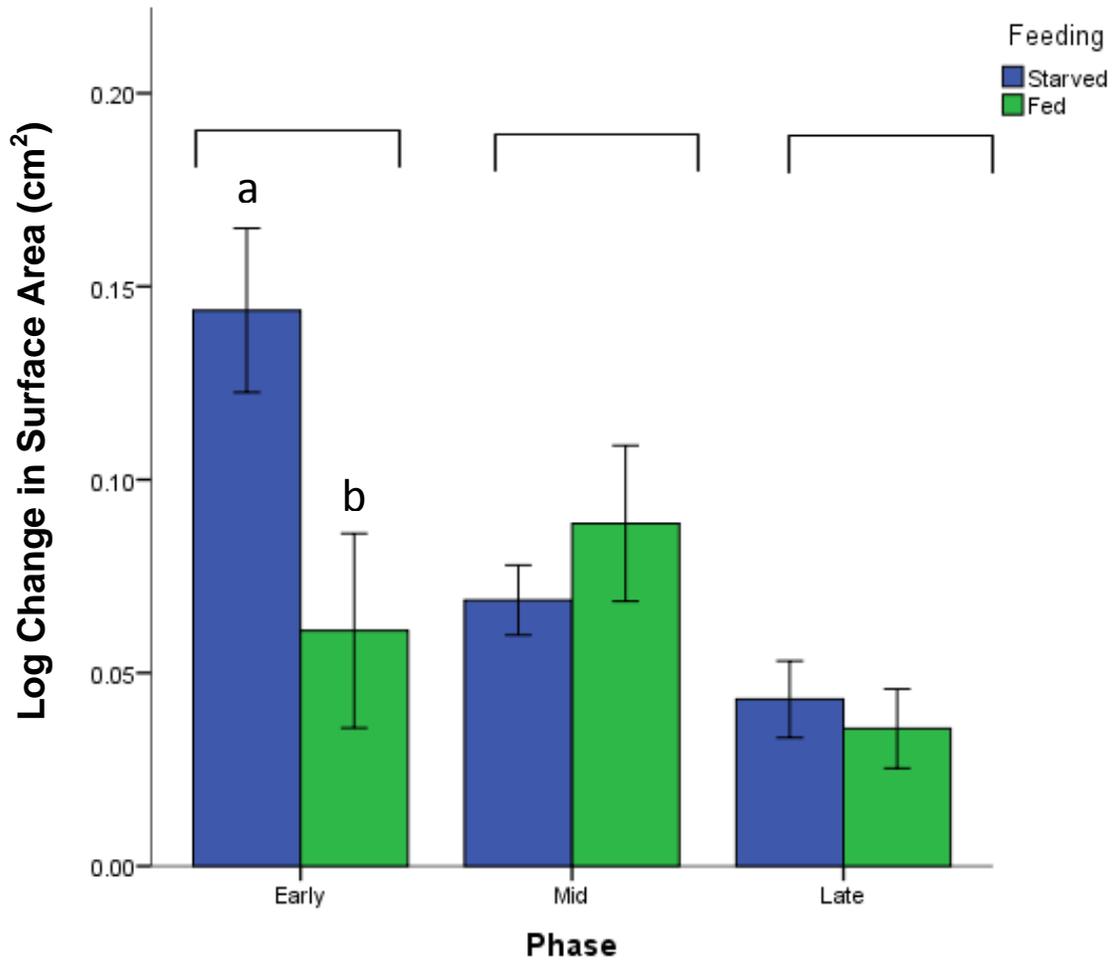


Figure 3.4: Mean (\pm SE) log change in surface area of *Rhodactis* sp. individuals at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation under different feeding treatments (fed = 1mL fine powder food every 3 days or starved). In the early phase starved individuals regenerated significantly more tissue than those that were fed ($p=0.003$). Food intake did not significantly affect the change in size in the mid and late phases of regeneration ($n=15-17$ per bar).

3.4.2 Zooxanthellae Density

For the initial trial on intrinsic factors, separate one-way ANOVAs were carried out to compare the effect of day, species, and size on zooxanthellae density (since data for the intrinsic factors failed Levene's test). Using this analysis, there was a statistically significant effect detected for all factors examined; day ($F_{(2, 67)} = 30.039$, $p < 0.001$), species ($F_{(2, 67)} = 5.693$, $p = 0.005$), and size ($F_{(1, 68)} = 15.534$, $p < 0.001$) on zooxanthellae density. Zooxanthellae density was highest prior to regeneration at day 0 (5.820 ± 0.115 log cells/g wet tissue); was reduced by the mid phase (4.996 ± 0.120 log cells/g wet tissue, $p < 0.001$) and reduced further when examined during the late phase (4.593 ± 0.108 log cells/g wet tissue, $p < 0.001$). However, in the replicate trial (not shown) this temporal pattern was not seen to be statistically significant. Large polyps had significantly more zooxanthellae per gram of tissue (5.459 ± 0.126 log cells/g wet tissue) than small polyps (4.815 ± 0.102 log cells/g wet tissue, $p < 0.001$) in the original trial (Figure 3.5) and this held true in the repeat trial as well ($p = 0.023$). *Ricordea yuma* (5.554 ± 0.131 log cells/g wet tissue) had significantly more zooxanthellae per gram of tissue than *Discosoma* sp. (4.876 ± 0.163 log cells/g wet tissue, $p = 0.005$) and, although not significant, more than *Rhodactis* sp. (5.042 ± 0.139 log cells/g wet tissue, $p = 0.05$) (Figure 3.6). The replicate trial did not test species.

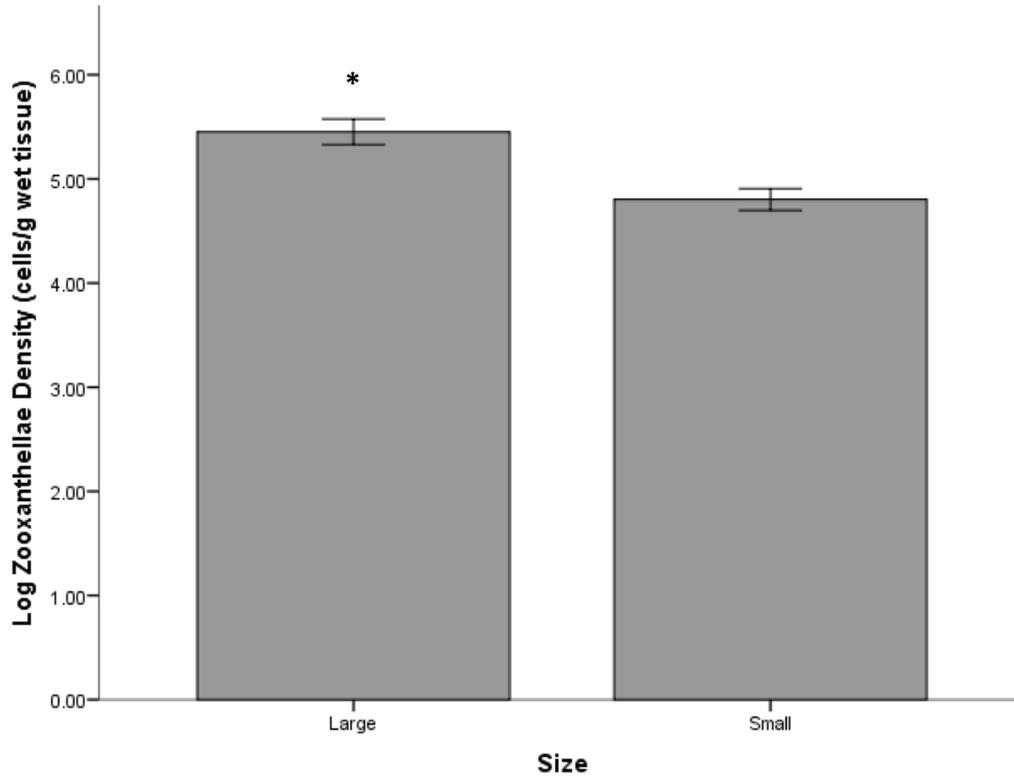


Figure 3.5: Mean (\pm SE) log zooxanthellae density in small (surface area $<5\text{cm}^2$) and large (surface area $\geq 5\text{cm}^2$) polyps of *Rhodactis* sp., *Discosoma* sp., and *Ricordea yuma*. Large polyps had significantly more zooxanthellae per gram of wet tissue than small polyps ($p < 0.001$) ($n=23-24$ per size).

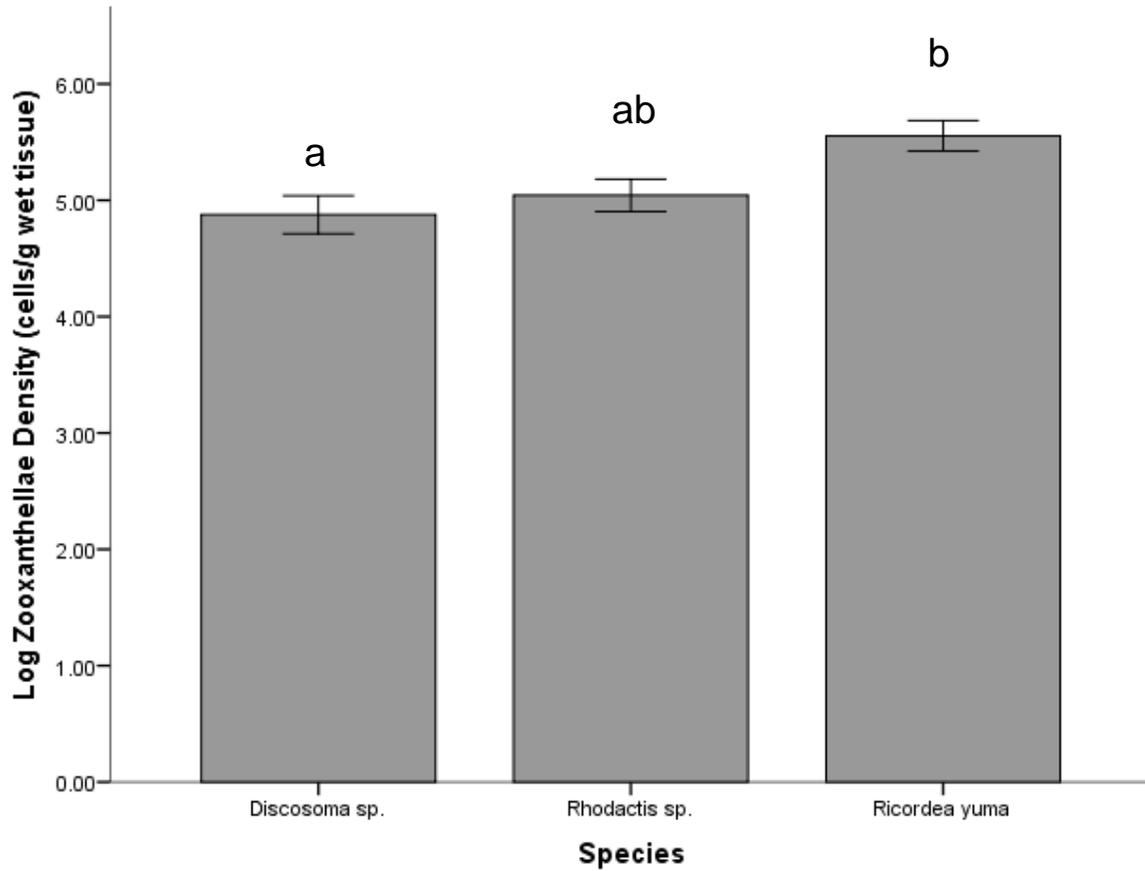


Figure 3.6: Mean (\pm SE) log zooxanthellae density in three different species. *Ricordea yuma* had significantly more zooxanthellae than *Discosoma* sp. ($p=0.005$) and though not significant, more than *Rhodactis* sp. ($p=0.05$) ($n=22-24$ per species).

The extrinsic trial data, from the original and replicate trials, could not be combined. They were each log transformed and analyzed by two-way ANOVA's. Temporal differences in zooxanthellae density were seen in the original extrinsic study ($F_{(2, 38)} = 5.300$, $p = 0.009$) where density was significantly higher pre-regeneration (day 0) (4.859 ± 0.193 log cells/g wet tissue) than post-regeneration (day 14) (4.269 ± 0.081 log cells/g wet tissue, $p = 0.023$), and although not significant, it was still higher than mid-regeneration (day 7) (4.507 ± 0.079 log cells/g wet tissue, $p = 0.299$) (Figure 3.7); however, no temporal differences were seen in the replicate trial ($p = 0.076$), similar to what was observed in the intrinsic trials. Lighting had no effect on zooxanthellae density in the original trial ($p = 0.368$); but did in the replicate trial ($F_{(1, 32)} = 12.975$, $p = 0.001$). Mid-regeneration polyps under high light conditions had more zooxanthellae (4.402 ± 0.137 log cells/g wet tissue) than those under low light (3.892 ± 0.137 log cells/g wet tissue, $p = 0.013$), and at the end of regeneration (day 14) polyps under low light had more zooxanthellae (4.106 ± 0.137 log cells/g wet tissue) compared to those under high light (3.625 ± 0.137 log cells/g wet tissue, $p = 0.019$) (Figure 3.7). Among lighting treatments, zooxanthellae density was significantly decreased when comparing day 7 to day 14 polyps held under high light ($p = 0.001$). Both original and replicate studies agreed that feeding did not have a significant effect on the zooxanthellae density, although there does appear to be an increase in density in fed polyps at day 7 and day 14 (Figure 3.8).

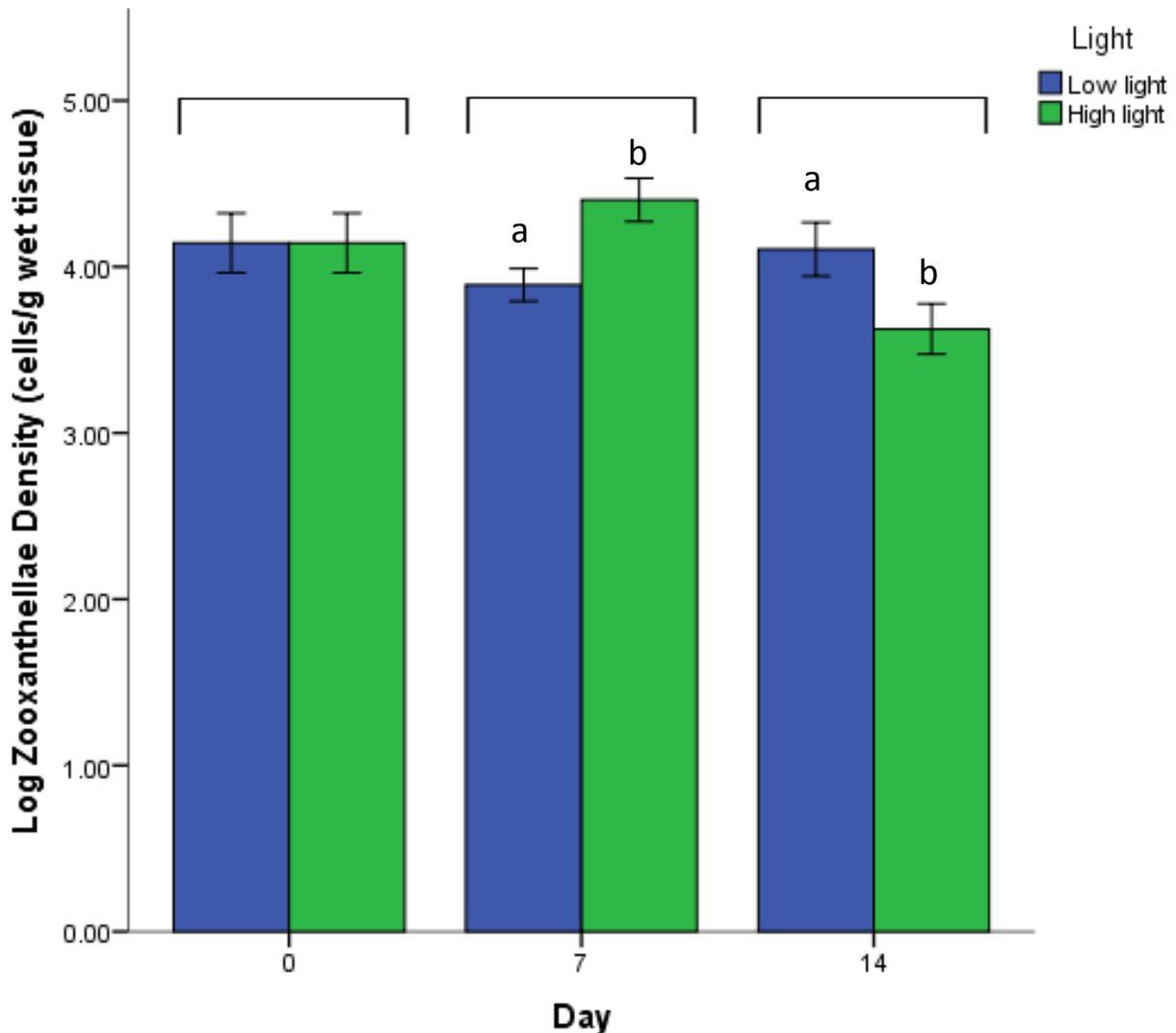


Figure 3.7: Mean (\pm SE) log zooxanthellae density in *Rhodactis* sp. under different light treatments (high = 120-150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and low= 30-60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) pre-fragmentation (day 0), mid-regeneration (day 7), and at the end of regeneration (day 14) post-fragmentation. At mid-regeneration, corals in high light have more zooxanthellae than those in low light ($p=0.013$). At the end of regeneration, corals under low light have more zooxanthellae than those in high light ($p=0.019$) ($n=5-8$ per bar).

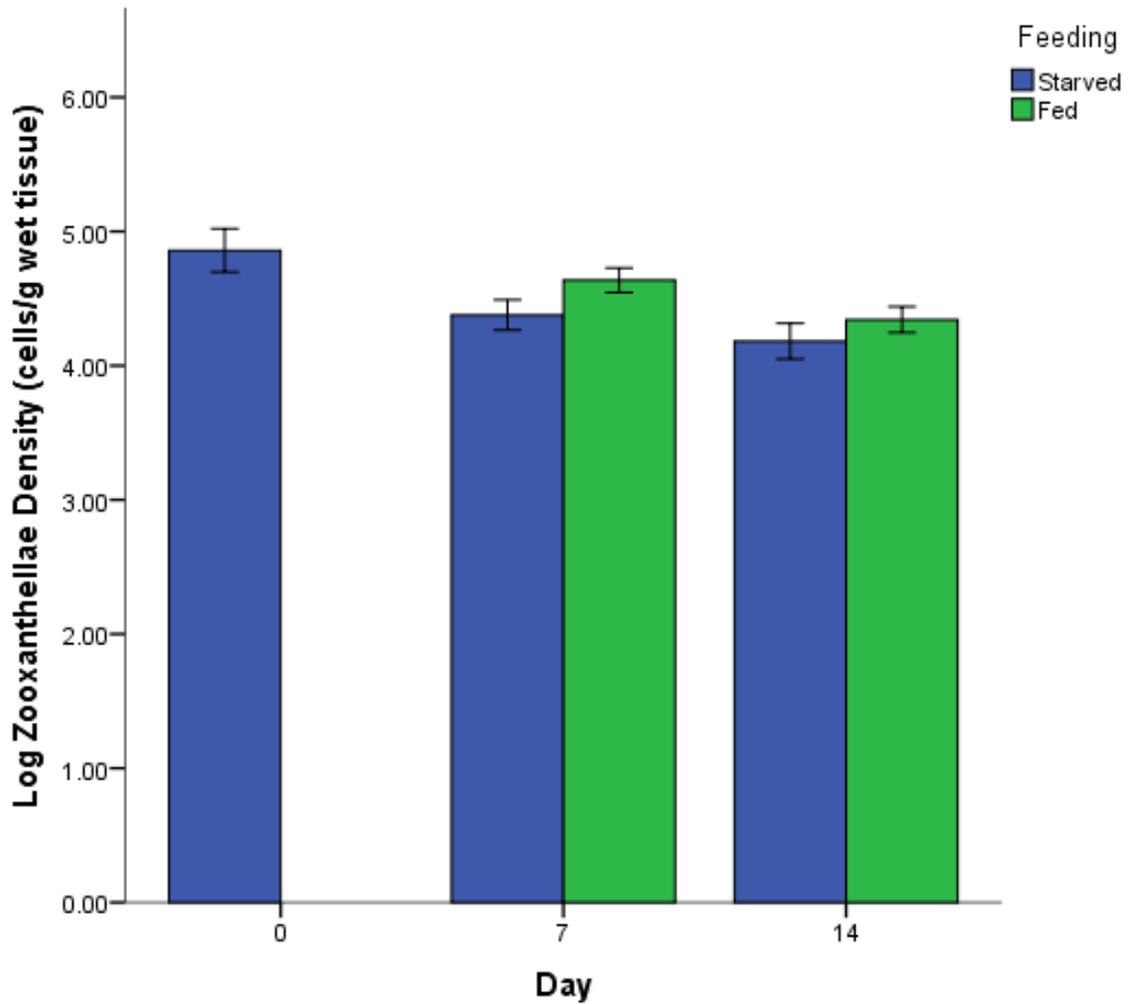


Figure 3.8: Mean (\pm SE) log zooxanthellae density in *Rhodactis* sp. under different feeding regimes; fed (1 mL of food every 3 days) and starved post-fragmentation. No significant difference was found among treatments; however, fed polyps appear to have greater zooxanthellae density (n=4 at time 0, and 12 at day 7 and 14).

3.5 Discussion

Regeneration in Corallimorpharia was unaffected by the intrinsic factors under the conditions used rejecting hypotheses 1 and 2, and individuals regenerated best under low light and no food rejecting hypotheses 3 and 4. Zooxanthellae densities significantly differed between polyp sizes, among species, and were significantly increased under high light. As well, though not significant, there was an increase in densities when polyps were fed. Regeneration happens quickly in Corallimorpharia (<2 weeks) compared to hard corals (4 weeks – 1 year) (Loya 1976, Kramarsky-Winter and Loya 2000, Oren et al. 2001, Denis et al. 2013), with the majority of the regenerative process occurring within the first six days. An early burst of regeneration is a common strategy in other species of coral (Loya 1976, Bak 1983, Meesters et al 1997, Kramarsky-Winter and Loya 2000). Regeneration must occur quickly to avoid algae settlement and the entrance of pathogens. Since most of the regeneration occurred in the early phase (day 2-6) in the present study, where the coral is presumably attempting to isolate itself from the environment, I will consider this to be the critical phase. In *Fungia granulosa*, individuals that did not recover by 8 weeks often never fully recovered during the spring season (Kramarsky-Winter and Loya 2000), suggesting factors affecting the early regeneration can have detrimental effect on the individuals' survival.

The lack of significance in regeneration rate between polyp sizes was unexpected. In *F. granulosa*, the greater the extent of damage (10<30<50%), the greater the difference in regeneration rate between size classes. Polyps of *F. granulosa* with 50% lesions showed the greatest difference in regeneration rate between large and small

individuals (Kramarsky-Winter and Loya 2000). Perhaps the 50% lesion size used in this study was not a factor contributing to the lack of significant difference found in the present study. Small Corallimorpharia individuals seem to be much hardier than that described for small individuals of other hard corals. In many studies, small individuals that were damaged experienced algal overgrowth and died (Loya 1976, Smith and Hughes 1999, Kramarsky-Winter and Loya 2000). Small corallimorpharians are less susceptible to death after fragmentation compared to small polyps/colonies of other hard coral species studied. A historically high rate of injury may have selected for increased energy reserves to carrying out regeneration, resulting in a consistent regeneration rate regardless of size. This response to a high injury rate has been proposed for soft corals where species regeneration differed at lower depths and was equal in shallow environments where they are more susceptible to damage (Wahle 1983, Henry and Hart 2005). Since the collection sites for the Corallimorpharia used in the present study were not known, including the depths or other environmental differences, this is highly speculative. This study suggests that Corallimorpharia regeneration acts independently of size. Size tends to relate to maturity, and reproductive state has the ability to affect the regeneration rate (Jackson and Hughes 1985, Kramarsky-Winter and Loya 2000). During gametogenesis *F. granulosa* was unable to completely regenerate no matter what the size of the individual or lesion (Kramarsky-Winter and Loya 2000). Reproductive state was not identified for the Corallimorpharia used in this study. It's possible that Corallimorpharia reach maturity at a smaller or larger size than 5 cm² surface area, and the two size classes used in this

study may have both had the same reproductive states represented, or one class had a combination of mature and immature polyps.

The lack of differences in regeneration rate between species may also be for the same reason; selection for consistent regeneration rates across species of tropical corallimorpharians. Coral morphology is considered to be a major determining factor in regeneration (Hall 1997). Corallimorpharia don't differ in morphology to the same extent as hard corals and the Corallimorpharia species chosen for the present study had very similar morphology. This may explain why they did not significantly differ in their regeneration rate. This was also in spite of the differences (sometimes significant) in zooxanthellae density between species. Another explanation might be that under the experimental conditions used there were no limiting factors such as light, food, and other water quality parameters to affect the rate of regeneration. Mortality was low during the present studies (i.e. intrinsic only 3 of 96 polyps) also suggesting that conditions were close to optimal.

Feed and light intake go hand in hand as they both provide the coral with nutrients required to carry out biological functions. Corallimorpharia regenerated faster under low light and starved conditions in the critical phase. There has been debate whether corals are more heterotrophic or autotrophic (Franzisket 1970, Lewis 1974). To name a few, *Pocillopora elegans*, *Porites compressa*, *Montipora verrucosa*, and *Fungia scularia* are autotrophic (Franzisket 1970), and *Montipora capitata* has the ability to be heterotrophic (Grottoli et al., 2006). Testing for heterotrophy versus autotrophy has not been done in the Corallimorpharia, and it might be interesting to see because feeding did not increase the rate of regeneration. In the future, measuring metabolism

and nutrient flux within the host during this critical phase would help to understand how food intake may be having a negative effect on regeneration.

High light can allow for greater densities of zooxanthellae (Kuguru et al. 2007). Since zooxanthellae provide the host with nutrients needed for biological functions (Muscatine et al 1981, Wooldridge 2010), more zooxanthellae would allow for more nutrient production and an increased regeneration rate. However, the present study rejects this hypothesis for *Corallimorpharia*. High lighting did allow higher zooxanthellae densities, but lower regeneration rates occurred in the critical stage of regeneration. This phenomenon has been observed in *Acropora muricata* where higher irradiance resulted in increased zooxanthellae and reduced regeneration rates. Growth rates remained high after injury and energy was not devoted to the regeneration process (Denis et al. 2013). Since corallimorpharians are single-polyp species, growth and regeneration cannot be measured at the same time, but they may still be devoting their energy elsewhere. Again, supplementary metabolism studies would help to confirm whether the high zooxanthellae densities are increasing the net photosynthesis.

The coral host has an energetic cost required to maintain symbiosis with the zooxanthellae including providing protection them from UV light, eliminating excess waste such as reactive oxygen species (ROS), and providing the symbionts with sufficient CO₂ (Wooldridge 2010). If the zooxanthellae densities are too high, the costs of maintaining symbiosis may be so high that the coral may be unable to properly carry out other biological functions. Feeding may have had a negative effect on regeneration in the early phase for similar reasons. Nitrogen enrichment has been seen to increase zooxanthellae densities in *Pocillopora damicornis* (Stimson and Kinzie 1991). Though

not significant, there was an increase in zooxanthellae densities in fed polyps in the Corallimorpharia. In both high light and fed polyps there was the same pattern of low regeneration in the early phase and a peak in regeneration in the mid phase. Perhaps the lag in regeneration can be explained by the coral acclimating to the higher zooxanthellae densities. Corals are able to expel their zooxanthellae and have done so in high nutrient water (Stimson and Kinzie 1991). In the case of high lighting there was a significant decline in zooxanthellae density at the end of regeneration. It is possible that the corals reacted to the high densities and expelled zooxanthellae to complete regeneration.

Intrinsic factors are not comparable between Corallimorpharia and Scleractina, nor are they among the orders. Understanding the intrinsic effects for different species or families must be studied before continuing to look at extrinsic factors in regeneration. We cannot make assumptions from other close relatives. The first 6 days post fragmentation is the critical stage in Corallimorpharia because this is where the greatest regeneration is occurring. Changes to the regenerative ability in the critical phase are of greatest interest. High light and high feed intake have negative effects on the critical phase in which corals are accompanied by an increase in zooxanthellae densities. Future work might consider looking at whether the tropical Corallimorpharia are more heterotrophic or autotrophic, as well as differences in conspecifics collected from different depths, and the size at which they reach maturity. As high light and high feeding had negative effects on regeneration, examination of the relationship between coral host and symbiont in high densities may also be of interest.

General Discussion and Conclusions

This thesis has provided insight into corallimorpharian regeneration for the first time; as well as providing a description of *Rhodactis* sp. normal histology. An understanding of normal histology allows for future comparisons, and quantitative and qualitative data on the regeneration process provides baseline data to allow an examination of the influence of environmental stressors. This research has raised many questions regarding corallimorpharian regeneration and normal histology. Determining whether corallimorpharians regenerate by morphallaxis, the migration of cells, or epimorphosis, proliferation of cells, is still to be determined. A study to label cells undergoing mitosis in both normal and regenerating polyps would help to determine if proliferation is occurring in the healing margin. In addition, amoebocytes are clearly experiencing a change during regeneration; however, their function during regeneration and whether or not they are phagocytic is unknown.

Investigations on histomorphology were challenging at times, particularly when making quantitative measurements. Several fixation methods had to be tested to identify the best one readily available to maintain cellular detail. Rupture of the gastrodermis was reduced in the chosen fixation method, but not eliminated. This made measurements of zooxanthellae density in the gastrodermal layer problematic. In addition, when carrying out the histological description of regeneration, the major changes to the tissue were occurring within approximately 700 μm (1 field of view at 200x magnification) from the healing margin. Measurements included 3 images and 3 fields of view from the healing margin. The inclusion of the 3 positions in my analysis

was to try to account for the more localized changes; however, there was still a lack of significance at this level. It might be interesting to make these measurements on an even smaller scale, using 400x magnification photos. Obtaining ideal sections for the description of regeneration was also a challenge. The leading tissue mass was difficult to catch in all sections. Five serial sections at 100 μm apart would have captured greater detail than using only two sections at 150 μm apart. When some sampled times were redone to improve sectioning, 5 levels were taken and it was discovered that closure begins from the bottom up. Deeper sections demonstrated enclosure of actinopharynx, whereas in shallower sections of the same individual the actinopharynx was still fused to the external epithelium.

This thesis also provided insight into optimal conditions to achieve the fastest regeneration rate in corallimorpharians. It is this work that provides a framework for any trials focusing on influences of environmental factors on regeneration, and the genomics and proteomics of regeneration. The early phase, days 2 – 6, was clearly the most critical phase of regeneration as polyps experienced the greatest increase in tissue during this time. It was discovered that intrinsic factors, including polyp size and species, did not significantly influence the rate of regeneration. Investigating reproductive state and how it relates to polyp size in the corallimorpharians, understanding whether there are difference in metabolism between size categories, and investigating the flow of nutrients from zooxanthellae to host in regeneration may provide insight to explain the phenomena observed in this work. Low lighting conditions and starving polyps were optimal for regeneration rate. Several questions remain; whether zooxanthellae type played a role in these results and understanding the

metabolism associated with the different light and feeding regimes might provide insight as to why regeneration is faster under a given set of conditions.

Challenges arose in determining the best method of measuring the rate of regeneration. Surface area can be problematic at times because the corallimorpharians are not always extended flat. There are often folds in the oral disc that a surface area taken from a two dimensional image does not properly account for. Wet weight measurements were attempted in one trial but this method was inaccurate (Appendix 3.1). Polyps actively take up and expel water naturally, and they would often expel water when lifted out of the tanks for measurements. An accurate weight was therefore not possible, so surface area was the preferred measurement. The gross morphology of corallimorpharian regeneration is different from scleractinians; they shrink in size post-injury whereas hard corals do not (personal observations). Surface area was a common method to measure regeneration in hard corals because it measures the lesion shrinkage over time. In Corallimorpharia changes in surface area measure the increase in size of the animal, and one challenge was to ensure only regeneration and not growth was measured. This is where the histological description of regeneration helped to ensure 14 days was a proper trial time. Closure did occur by 4 and 8 d, so some growth may be measured, but likely only in the late phase. Splitting the 2-week period into early mid and late phases was helpful for ensuring that growth is not grouped with regeneration. It's likely that the early phase is mostly just regeneration, whereas mid and late phases may have included growth. However, these times cannot be ignored because in the high light and fed polyps the greatest change in surface area was in the

mid phase suggesting there may have been a lag in regeneration that would have been missed if measurements were made only until 6 d.

The following conclusions can be drawn from:

Chapter 2:

1. The microscopic regeneration of the corallimorpharian *Rhodactis* sp. follows a wound healing approach of tissue extension, sealing, tissue organization, and structural organization.
2. Infiltration of amoebocytes was not observed in the regeneration process; however, a change in morphology of amoebocytes was found.
3. Individual variation in the speed of regeneration does exist as fusion occurred from 2 to 4 days, and wound closure ranged from 4 to 8+ days post-fragmentation.

Chapter 3:

1. Regeneration rate was equal among three corallimorpharian species (*Discosoma* sp., *Rhodactis* sp., and *Ricordea yuma*) and two polyp size categories (small, surface area $< 5 \text{ cm}^2$, and large, surface area $\geq 5 \text{ cm}^2$).
2. High light intensity increased zooxanthellae density and decreased rate of regeneration in the early phase (day 2-6). Low light intensity was optimal for achieving the fastest regeneration in the early phase.
3. The starvation of polyps from additional particulate food sources resulted in faster regeneration rates relative to individuals that were fed.

4. The model to be used in future work will be species and size independent, and the chosen corallimorpharian will be held under low light intensities (30-60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and not fed for the 14 day trial.

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Appendices

Chapter 2 Appendix

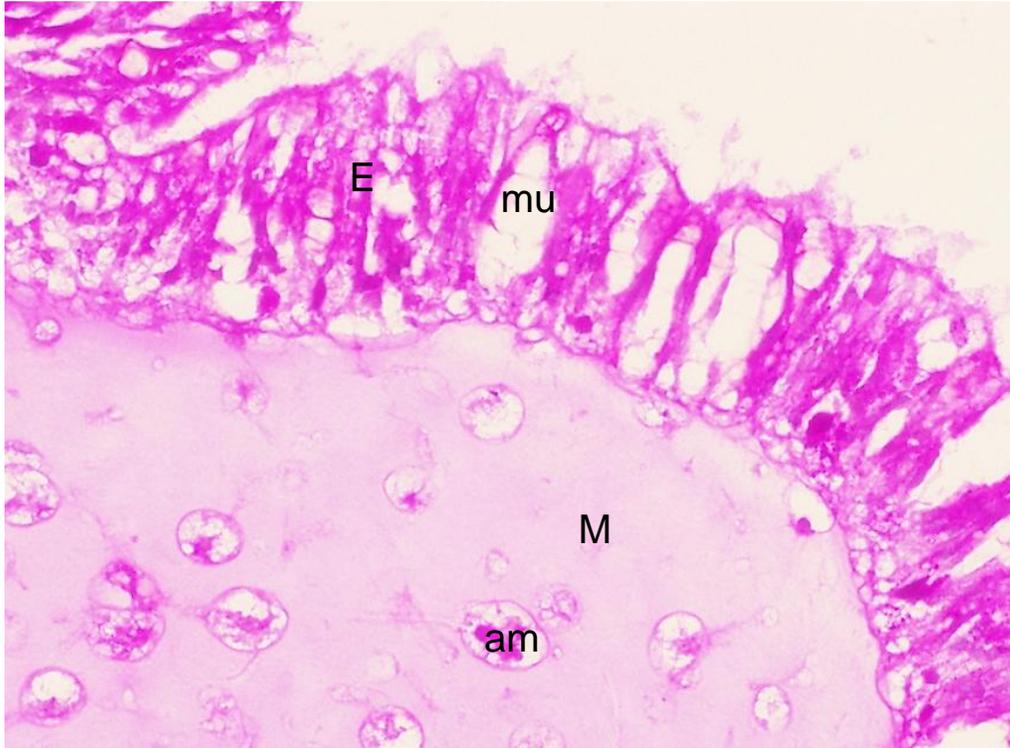
2.1 Fixation

Six fixation regimes were carried out with three replicates per treatment. The treatments included two fixatives, Bouins (Ricca Chemical Company, Arlington TX) and Z-Fix (Anatech Ltd, Battle Creek MI), with three prep methods; dose relaxation (in 7% MgCl₂), drip relaxation (50mL 14% MgCl₂ into 50mL seawater), or unrelaxed/direct fixation. Samples that were relaxed in MgCl₂ were allowed 3 h in the mixture under regular LED light regime. Polyps were then placed into their designated fixative and fixed for 24-48 h. Polyps were routinely processed for histology (Animal Health Lab, University of Guelph), embedded in paraffin, two 6 µm sections were taken 200 µm apart and stained with H&E. A good fixative method was based on the highest cellular detail of amoebocytes and epithelial cells, as well as minimal displaced nematocysts within the gastrodermis.

Z-Fix samples had shrunken nuclei in the epilethium, poor amoebocyte detail and increased sloughing of cells in the gastrodermis. The Bouins fixative demonstrated better fixation and the two relaxation techniques proved to have a negative effect on fixation as the mesoglea appeared shrunken as well as necrosis and sloughing of

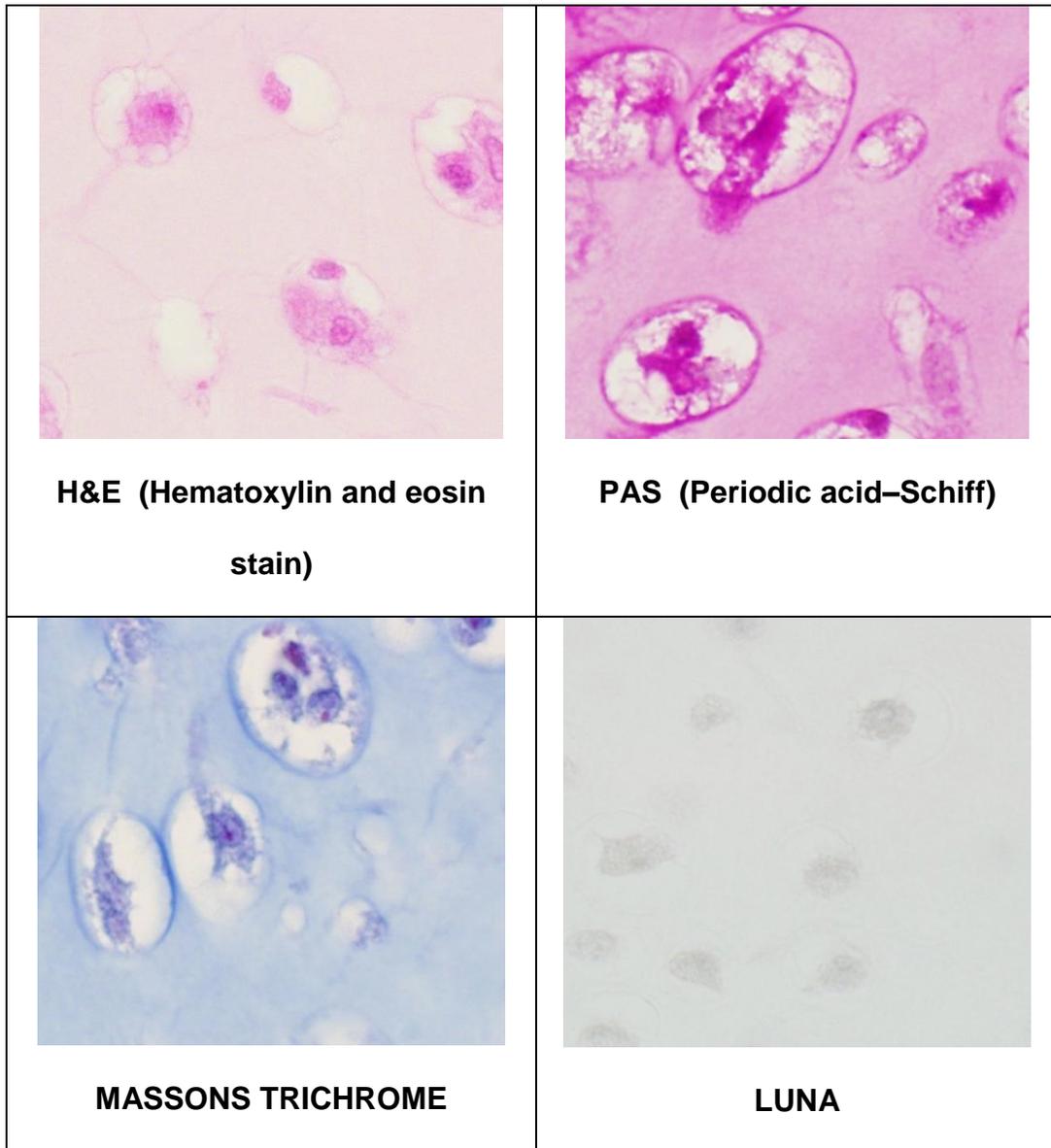
epithelial cells was observed. Bouins direct fixation (24 h) was chosen as the superlative fixation method for Corallimorpharians.

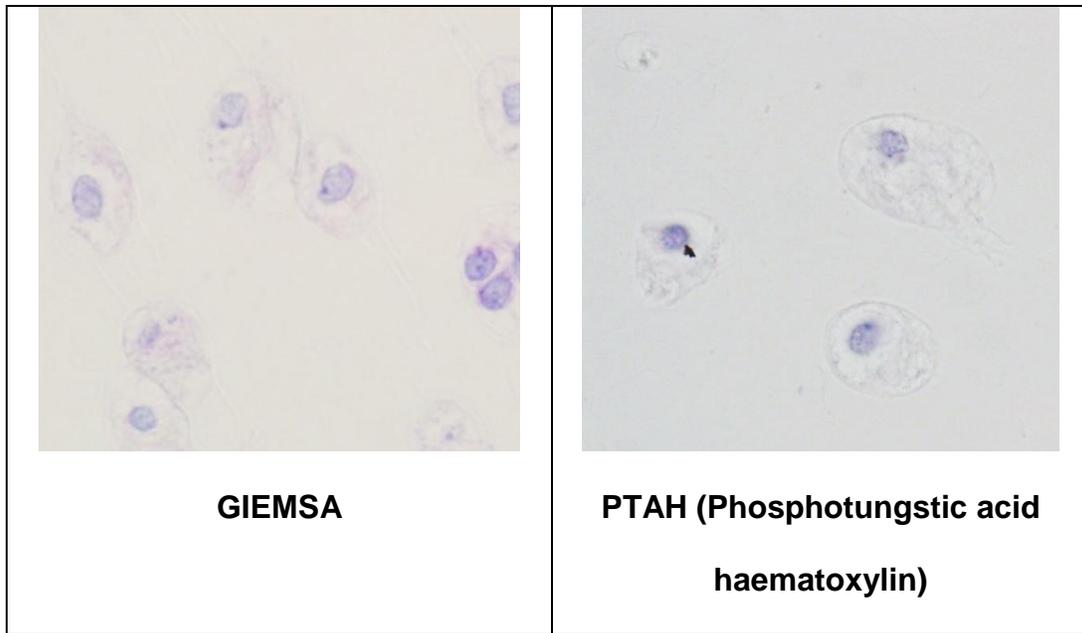
2.2 Special Stains



Healthy *Rhodactis* sp. tissue stained with PAS. Mucus in the epithelium did not preferentially stain. Epithelium (E), mesoglea (M), amoebocytes (am), mucocytes (mu); 400x PAS.

In attempt to determine a way to identify amoebocytes outside of the mesoglea, and differentially from epithelial cells a number of stains were tested. None were helpful in selectively staining amoebocytes:





Chapter 3 Appendix

3.1 Regeneration by Weight

Fragments were weighed to the nearest 0.01 g on days 2, 6, 10, and 14 post-fragmentation (n=4). Weights were used to calculate the change in size in the early (day 2-6), mid (day 6-10), and late (day 10-14) phases. Data was tested for normality (Shapiro-Wilk $\alpha > 0.05$), sphericity (Mauchly's test $\alpha > 0.05$), and checked for outliers. Following that a two-way repeated measures ANOVA was performed to identify whether phase, size, light, and feeding had an effect on the regeneration rate. The test on size used day 2 weights as a covariate to account for initial size differences.

Size did not have a significant effect on the rate of regeneration in any phase ($p=0.362$, Figure 1 A), nor did any phase ($p=0.125$, Figure 1 B) in those polyps. The

extrinsic factors ANOVA demonstrated that there was no effect of lighting ($p=0.571$, Figure 2 A) or feeding ($p=0.492$, Figure 2 B) on regeneration rate at any phase, but regeneration rate did significantly differ among phases ($F_{(2, 22)}=4.823$, $p=0.018$, Figure 2 C). A least significant difference post hoc determined that polyps in the early phase regenerated significantly more (0.251 ± 0.09 g) than those in the mid (0.045 ± 0.042 g, $p=0.048$) and late phase (-0.147 ± 0.095 , $p=0.028$) (Figure 2C).

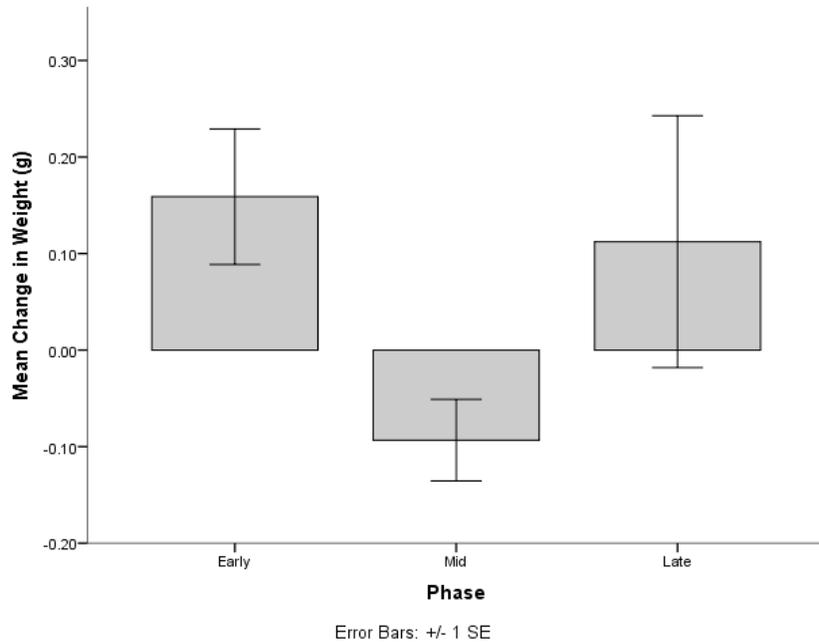
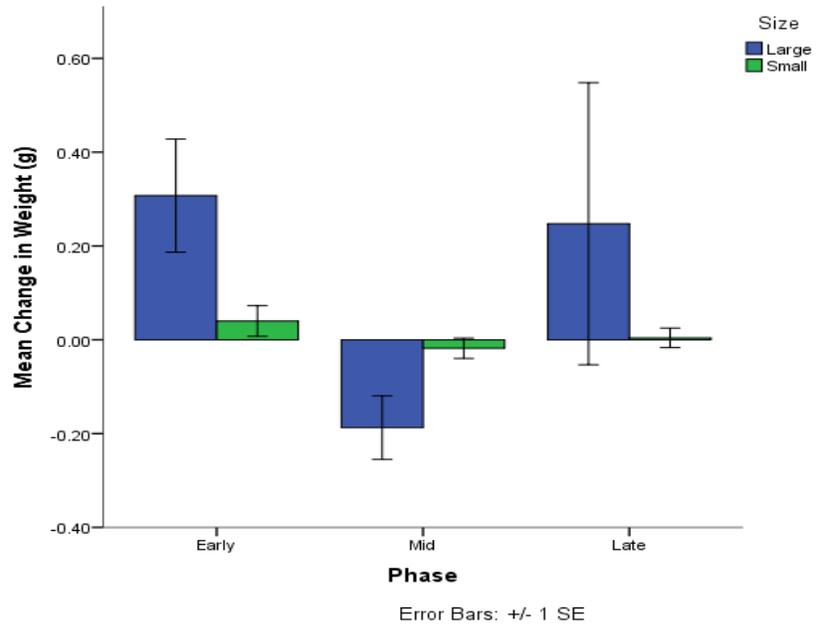


Figure 1: Mean change in weight of regenerating *Rhodactis* sp. individuals at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. A) Sizes (Small <5 cm² surface area, Large ≥5 cm² surface area) did not significantly vary in regeneration rate among any phases. B) Polyps did not significantly differ in regeneration rate among the three phases.

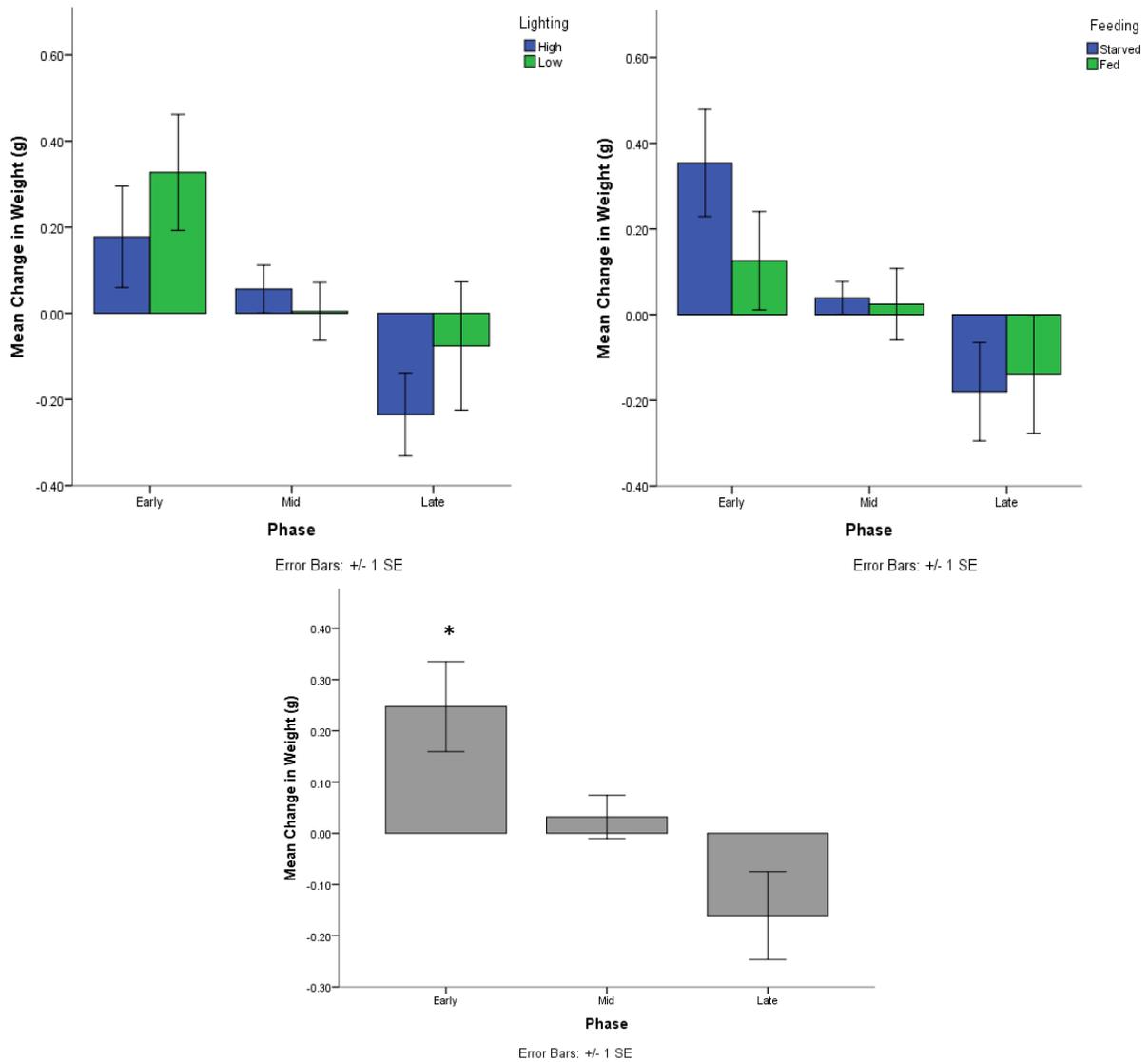


Figure 2: Mean change in *Rhodactis* sp. weight at early (day 2-6), mid (day 6-10), and late (day 10-14) phases post-fragmentation. A) Polyps did not significantly differ in regeneration rate among phases under high ($120\text{-}150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or low ($30\text{-}60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) lighting conditions. B) Feeding had no effect on regeneration rate. C) Polyps regenerated significantly more weight in the early phase than the mid and late phases.