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Molecular Bases of Soft Coral Reproduction

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in

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by

Wiebke Wessels

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DEDICATION

To my family, who always believed in me

STATEMENT OF SOURCES

I certify that the presented thesis

Molecular Bases of Soft Coral Reproduction

is, to the best of my knowledge and belief, original and my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Wiebke Wessels

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THESIS ABSTRACT

The early development of the hard coral *Acropora millepora* has been described at both the morphological and molecular levels, and comparative studies are under way for other Scleractinia. Complementary data on Octocoral embryogenesis are, however, essential for a broader understanding of the different developmental strategies in Cnidaria and their evolution. Although the morphology of soft coral embryogenesis has been previously described, data are scarce and molecular data complementing the morphology are missing. This thesis involves the investigation of reproduction and early development in soft corals, using *Lobophytum pauciflorum* as model. As in the majority of soft corals, *L. pauciflorum* colonies are either female or male. The time of reproduction approximately coincides with the annual synchronised mass spawning of hard corals on the Great Barrier Reef (GBR), although in favourable years studied, it occurred a lunar month earlier. The process of gametogenesis is longer in the gonochoric soft coral than in the hermaphrodite hard coral *A. millepora* or the gonochoric hard coral *Fungia concinna*. However oogenesis is completed within a year, which is shorter than has been reported for a conspecific population in Taiwan. In addition, bidirectional sex change was observed in *F. concinna*, suggesting that this trait could also be a predominant reproductive feature in fungiid corals.

After studying the gametogenic cycle of *L. pauciflorum* on the GBR and identifying the time point of spawning as 4 − 5 nights after the austral spring full moon, investigation of early development was possible. Following mixing of eggs and sperm, the first obvious signs of successful fertilisation occurred at the 16 − 32 cell stages. Through a series of cell divisions leading to irregular shapes, embryos developed into a swimming planula within 48 hours of fertilisation. Planulae then stayed in the water column for up to a week before settling and developing into a primary polyp with 8 tentacular buds in the 24 hours after settlement. Within the first week of metamorphosis, sclerites were visible inside the polyp tissue. Early development in *L. pauciflorum* appears to be slower than in *A. millepora*, and it was not possible to identify developmental stages corresponding to those of hard corals on the basis of morphology. Some similarities with hard corals were observed in terms of timing of expression of genes involved in calcification.

In order to extend the knowledge on soft coral microbial communities, microbes associated with the entire colony were also investigated in *Lobophytum pauciflorum*. In a first approach the possibility of sex−specific differences in the core microbiome of *L. pauciflorum*

was investigated. The profile of ribotypes present in at least 51 % of the samples was similar to the general profile when all ribotypes are considered. The microbial community in *L. pauciflorum* was dominated by Spirochaetes−related ribotypes, though a−Proteobacteria were the second most abundant class. Further, microbial profiles were very similar between male and female colonies in terms of highly abundant ribotypes, although sequences that cannot be assigned to any known bacteria were significantly more abundant among female than male colonies. Some sex−specific differences were detectable in ribotypes of low abundance. However, at class and family level, these differences do not persist, suggesting that the different ribotypes exhibit similar functions. The high abundance of unknown ribotypes provides the potential for discovery of novel bacterial strains with potentially different functions. In a second study, the effect of environmental stresses on the microbial community of *L. pauciflorum* was investigated. Microbial profiles of *Lobophytum* fragments exposed to 1) elevated temperatures, 2) reduced pH and 3) a combination of elevated temperatures and reduced pH for 12 days did not vary from control conditions. However, a clear pattern of colony specific microbial profiles at ribotype level emerged. A Spirochaete−dominated microbial community has only recently been identified in a gorgonian species in the Mediterranean Sea and deserves further characterisation. Combining the results of both studies showed that the microbial community of *L. pauciflorum* is conserved, with limited colony specific variability. Further, the microbial community appears robust to short term stresses, however the effect of longer−term stress deserves further investigation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 General Introduction

In many ways, corals are the principal components of reef ecosystems. Hard corals (order Scleractinia) are the major architects of coral reefs and soft corals (order Alcyonacea) provide important habitat structures, e.g. for reef fish. These critically important groups of animals are in decline as reefs worldwide suffer the impacts of climate change and other anthropogenic challenges. The molecular details of how climate change and pollution affect coral health are largely unknown at this time, but the molecular tools to enable a better understanding of these processes are becoming available. Both Scleractinia and Alcyonacea are classified in the Anthozoa, the sister group of the Medusozoa (containing the other cnidarian classes) (Figure 1.1). The phylum of Cnidaria itself has diverged before the radiation of the Bilateria. These organisms therefore hold a special position in the evolution of complexity. Due to their basal phylogenetic position within the Eumetazoa, these organisms comprise a useful group for understanding many aspects of animal evolution, including the molecular mechanisms controlling early development, genomic characteristics, and the evolution of the nervous and immune systems.

1.1 A brief note on the anthozoan subclass Octocorallia

Cnidaria are the closest phylum to the Bilateria within the Metazoa, emerging after the divergence of Porifera, probably about 740 Ma as suggested by Park and colleagues (2012). This phylum is composed of 5 classes, Anthozoa, Cubozoa, Hydrozoa, Scyphozoa and Staurozoa (Figure 1.1) and major taxa diversified prior to Cambrian (~ 530 Ma). While the medusa stage is important in the life history of Cubozoa, Hydrozoa, Scyphozoa and Staurozoa, this stage is missing in Anthzoa, though some hydrozoan species lack this stage as well. The presence of the characteristic medusa stage led to the introduction of the subphylum Medusozoa, excluding the Anthozoa. Anthozoa is composed of two major subclasses, Hexacorallia (anemones, scleractinian corals and black corals) and Octocorallia (soft corals, gorgonians and sea pens). Most of the corals in shallow waters, irrespective of the subclass, are an association between a coral host and a photosynthetic dinoflagellate from the genus *Symbiodinium*. This symbiotic relationship is obligate for the so called ‘zooxanthellate’ or ‘hermatypic’ coral host, though corals are able to survive a short period of time without their photosynthetic partner. The photosynthetic symbiont provides a large proportion of the energy budget of the coral host

(Falkowski *et al.*, 1984), though octocorals are suggested to depend less on the nutritional supply (Fabricius and Klumpp, 1995).

Under classical taxonomy, based on morphological features within the Anthozoa, the relationship between Hexacorallia and Octocorallia is considered monophyletic (Won *et al.*, 2001; Daly *et al.*, 2007). This view is currently under debate as depending on the sequences (mitochondrial sequences, mitochondrial genomes or entire transcriptomesƒgenomes) and the number of species included the relationship could also be paraphyletic with the Octocorallia being a sister group to the Medusozoa (Park *et al.*, 2012; Kayal *et al.*, 2013; Stampar *et al.*, 2014; Figueroa and Baco, 2015; Zapata *et al.*, 2015). Due to this ongoing discussion it is hard to estimate the divergence between these two subclasses, possibly about 250 Ma with the last common ancestor being a survivor of the Permian–Triassic mass extinction as suggested by Park and colleagues (2012). As the above mentioned studies favouring the paraphyletic view could not reject the anthozoan monophyly, I share the view of Zapata and colleagues that morphological features are synapomorphies rather than convergent (Zapata *et al.*, 2015) and support the monophyletic relationship between Octocorallia and Hexacorallia (Figure 1.1).

Regardless of their evolutionary relationship, Hexacorallia and Octocorallia are differentiated by the symmetry in their body. Polyps of Hexacorallia have a 6–fold symmetry whereas octocoral polyps have an 8–fold symmetry. Another crucial difference lies in the form in which calcium carbonate is deposited in the two groups. Hexacorallia were found to secrete aragonite crystals whereas calcite predominates in Octocorallia (Clarke and Wheeler, 1922). When present in the latter, calcite crystals are located in the mesoglea of the polyp body and in some species also in the axis of the colony, forming bone–like structures. These species–specific constructs are suggested to play a role in physical support and defence against predators (Van Alstyne *et al.*, 1992). In Hexacorallia, aragonite crystals form an exoskeleton providing structure and habitat for other reef organisms and thus making scleractinian corals (hard corals) the major reef builders. Due to the lack of an exoskeleton, octocorals are often not considered as reef building corals. However some *Sinularia* species can contribute to the reef structure by consolidating sclerites at the colony base to form spiculite and amorphous calcium carbonate (Jeng *et al.*, 2011).

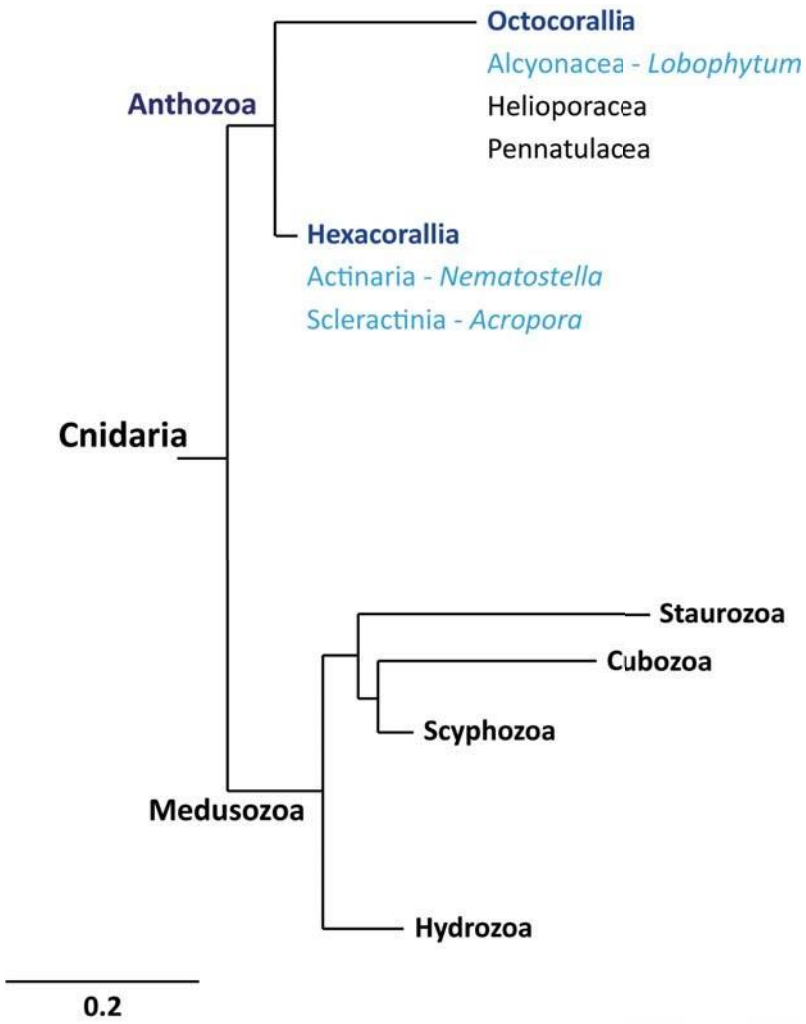


Figure 1.1 Phylogenetic relationship among the classes of Cnidaria (summarised after Zapata *et al.*, 2015).

Octocoral sclerites are also of great taxonomic significance, as taxa have been described primarily based on sclerite structure (Bayer, 1956; Fabricius and Alderslade, 2001). The development of molecular biological techniques has allowed the combination of morphological and molecular tools to determine phylogenetic relationships within the Octocorallia (e.g. McFadden *et al.*, 2006). In addition to common mitochondrial and ribosomal markers, the discovery of an octocoral specific mitochondrial gene allowed extension of these analyses (McFadden *et al.*, 2010). However, to date the phylogenetic relations between and within the orders of the Octocorallia could not clearly be resolved and it is hoped that additional genome and transcriptome data can provide enough characters to resolve these relationships (McFadden *et al.*, 2010). The currently consensus is that the Octocorallia comprises 3 orders: Pennatulacea or sea pens, Helioporacea or blue corals and Alcyonacea, which includes the soft corals, gorgonians and stoloniferous forms (Fabricius and Alderslade, 2001; Daly *et al.*, 2007; McFadden *et al.*, 2010).

Octocoral phylogeny might still be unclear and molecular studies are scarce to date to clarify evolutionary relationships. Irrespective of the phylogenetic relationships among octocorals, chemical analysis on alcyonacean complementary (secondary) metabolites have been of broad interest. Common secondary metabolites include terpenoid compounds, of which some have been shown to be toxic to fish or show antibacterial properties (Coll and Sammarco, 1983; Zhao *et al.*, 2013). Soft corals use secondary metabolites for predator defence (Coll et al., 1982; Coll and Sammarco, 1983; Sammarco et al., 1987) and competition for space with harmful effects on hard corals (e.g. Sammarco et al., 1983; Sammarco et al., 1985; Atrigenio and Aliño, 1996). These secondary metabolites are also an important component of soft coral eggs, possibly protecting them from predation (Michalek−Wagner and Willis, 2001b)

1.2 Reproduction

Sexual reproduction of scleractinian corals has been extensively studied at the ecological level (reviewed in Harrison and Wallace, 1990) but the underlying molecular mechanisms are largely unknown. Two different modes of reproduction predominate in corals: broadcast spawning and brooding. Brooding generally occurs internally in the maternal polyp; however, in some soft corals brooding can also occur externally on the surface of the maternal colony. Broadcast eggs are quickly fertilized, which is in line with the generally rapid development. The vast majority of scleractinian corals are described as simultaneous hermaphrodites (Fadlallah, 1983; Harrison and Wallace, 1990), whereas most alcyonacean corals have separate sexes (Kahng *et al.*, 2011). In contrast to the majority of Scleractinia, solitary hard corals such as fungiids are gonochoric (Fadlallah, 1983; Krupp, 1983; Kramarsky–Winter and Loya, 1998). The observation of sex change in *Fungia repanda* and *Ctenactis echinata* described recently (Loya and Sakai, 2008), extended the reproductive repertoire for Fungidae to being sequential hermaphrodites. In both *Fungia scutaria* and *C. echinata,* female individuals are consistently larger than males, implying that these species are protandrous sequential hermaphorites (Kramarsky–Winter and Loya, 1998; Loya and Sakai, 2008).

During the week after a full moon in late Southern hemisphere spring, over 130 scleractinian species were observed to spawn synchronously at the Great Barrier Reef (GBR) (Willis *et al.*, 1985) and spawning of more than 30 species was described to occur within hours on the same night at a single location (Willis *et al.*, 1985; Babcock *et al.*, 1986). Some inshore populations were recorded to spawn one lunar month before populations of the same species on offshore reefs. When the full moon occurs in the first half of the months of October and November, spawning is often split over two months, with some colonies releasing gametes one month before the remainder of the population (Willis *et al.*, 1985). The majority of soft corals were described to reproduce in a synchronized events coinciding with the mass spawning of scleractinian corals (Alino and Coll, 1989).

1.2.1 Sex determining factors in corals

Despite the identification of corals that have separate sexes, sex−determining factors in corals are still unknown. In contrast to most vertebrates, cnidarians do not have sex specific chromosomes. It is rather suggested that the sex is determined through differential expression of specific 'sex determining genes', e.g. *DM*, *Sox* or *FoxO,* which are thought to also be key players in gametogenesis.

Although sex determination is achieved in very different ways across the animal kingdom, members of the DM family of transcription factors have been implicated in sex determination or differentiation in many metazoans. The DM domain is a DNA binding motif first identified in the *Drosophila melanogaster* Doublesex (DSX) protein and the MAB−3 protein in *Caenorhabditis elegans* (Raymond *et al.*, 1998). Both proteins were found to bind similar DNA sequences (Erdman *et al.*, 1996; Yi and Zarkower, 1999) and exhibit a regulatory function in sex−specific neural development and yolk protein synthesis. In *C. elegans,* MAB−3 can be replaced by the male form of DSX (Raymond *et al.*, 1998). These structural and functional similarities suggest a common evolutionary origin of this mechanism for sexual development (Raymond *et al.*, 1998; Yi and Zarkower, 1999). In the swallowtail butterfly *Papillo polytes,* Doublesex was identified as single supergene in sex−specific mimicry, contrasting the traditional view of 'a supergene as tightly linked cluster of loci' (Kunte *et al.*, 2014). The expression of female isoform was characteristic on a temporal and spatial scale, *i.e.* increasing expression throughout development and in body and wings, respectively. Single nucleotide polymorphisms, which were mainly identified in the first exon, might lead to significant differences in the secondary and tertiary structure (Kunte *et al.*, 2014).

For Cnidaria, the DM domain was identified in the staghorn coral *Acropora millepora* with a high sequence similarity to the human, fly and nematode orthologs. This scleractinian DM gene encodes a number of transcripts, at least two of which encode proteins with a DM domain (Miller *et al.*, 2003). Consistent with a role in sexual development, expression of the coral DM gene correlated with gamete differentiation, in time (October maximum) and in space (branch tip maximum). Unfortunately, the hermaphroditic nature of *A. millepora*, severely complicates further investigation of the role of this DM gene in sex determination. For the gonochoric scleractinian *Euphyllia ancora,* the expression of a specific DMRT sequence was correlated with sex and reproduction (Chen *et al.*, 2016). *EaDmrtE* mRNA was enriched in the female germ cells during the spawning season but also in unfertilised mature oocytes and newly formed zygotes. These expression patterns suggest roles in oogenesis and early

development as a maternal factor (Chen *et al.*, 2016). Further, a recent study was able correlate the onset of gametogenesis with the expression of an *E. ancora* homolog of the *Drosophila* germline marker *vasa*. The coral homolog was detected in oogonia and spermatogonia and early stages of gametes but not in later developing or mature gametes (Shikina *et al.*, 2012).

In another member of the anthozoan subclass Hexacorallia, the starlet sea anemone *Nematostella vectensis,* sex−specific miRNA patterns were identified (Moran *et al.*, 2014). While 18 male specific miRNAs were identified, only a single miRNA was enriched in female samples. The identification of the targets of these sex−specific miRNAs remain to be determined (Moran *et al.*, 2014). Sex−specific miRNAs have, however, been identified in mammals (Ciaudo *et al.*, 2009) and flatworms (Hao *et al.*, 2010; Marco *et al.*, 2013) and may also exist in birds (Luo *et al.*, 2012). Similar expression patterns of miRNAs in *N. vectensis* and Bilateria highlight the potential role for miRNAs in cell or body region differentiation in the sea anemone (Moran *et al.*, 2014). At present, no data are available for corals.

1.2.2 Gametogenesis

The term gametogenesis describes the production and maturation of reproductive output. The duration of one gametogenic cycle varies among hard corals but is generally thought to be less than one year (reviewed in Harrison and Wallace, 1990). Regardless of the mode of reproduction, oogenesis generally precedes spermatogenesis in both simultaneous hermaphrodites and gonochoric species (Szmant–Froelich *et al.*, 1980; Szmant–Froelich *et al.*, 1985; Kojis, 1986; Szmant, 1986; Kramarsky–Winter and Loya, 1998). The length of the oogenic cycle was found to be more variable between species (about 3 – 11 months) than is the spermatogenic cycle (6 – 12 weeks) (Kojis, 1986; Szmant, 1986; Kramarsky–Winter and Loya, 1998). However, among Caribbean reef corals, polyp size and morphology do not correlate with reproductive patterns (Szmant, 1986). The onset and duration of gametogenesis does not differ significantly between gonochoric and hermaphroditic hard corals. Within the Faviidae, for example, the duration of oogenesis differs greatly between the simultaneous hermaphrodites *Montastrea annularis* (4 – 5 months) and *Diploria strigosa* (up to 9 months), whereas in the gonochoric species *Montastrea cavernosa* oogenesis occurs on a similar time frame to *D. strigosa* (Szmant, 1986). Szmant (1986) reported that two gonochoric hard corals, *Siderastrea siderea* and *Dendrogyra cylindrus*, can complete their oogenic cycles within 3 months. For *Fungia granulosa* and *F. scutaria*, for example, oogenesis was observed to take 7 – 8 months in the northern Red Sea and spermatogenesis 8 – 12 weeks (Kramarsky–Winter and Loya, 1998). In *Acropora spp.* oocytes were observed 9 – 11 months prior to spawning, whereas spermatogenesis is reported to take about 6 – 8 weeks (Kojis, 1986; Szmant, 1986). Zooxanthellate, hermatypic, corals were found to have optimum growth rates around the summer ambient temperatures of their natural habitat (Shinn, 1966; Jokiel and Coles, 1977). Hermatypic corals often largely rely on their endosymbionts for energy supply (Falkowski *et al.*, 1984) and a large proportion of photosynthetic products is also used in oogenesis in *Stylophora pistillata* (Rinkevich, 1989). Onset of gametogenesis is therefore also likely to depend on energy resources and spawning time. For a spawning in the early Australian summer, corals on the GBR would have to initiate the costly process of oocyte production at the end of the summer before the water cools down in order to complete the oogenic cycle.

On the GBR, alcyonacean corals were found to reproduce in a synchronized spawning events coinciding with the mass spawning of scleractinian corals (Babcock *et al.*, 1986; Alino and Coll, 1989). Diverging from the dominance of hermaphroditism in Scleractinia, soft corals are mainly dioecious, as in the case of solitary hard corals such as members of the Fungiidae (Kahng *et al.*, 2011). In contrast to hard corals, gametogenesis in soft corals has received only

limited attention, although studies on reproduction have increased over the last twenty years (Fan *et al.*, 2005; Yeung and Ang, 2008; Simpson, 2009; Nonaka *et al.*, 2015). As in hard corals, alcyonacean corals have two major reproductive traits: broadcast spawning of gametes with external fertilisation and development, and brooding of planulae either externally or internally (Benayahu, 1997a). Soft coral oocytes are characterised by large yolk content (Kruger *et al.*, 1998; Orejas *et al.*, 2002). However, the size of mature oocytes is highly variable between species (Lasker and Kim, 1996; Kruger *et al.*, 1998; Gutiérrez−Rodríguez and Lasker, 2004). The inter−specific variation in oocyte size might be due to several factors such as the size of the polyp and its location within the colony (Brazeau and Lasker, 1990) and the reproductive strategy and fertilisation success of the species, including the mode of larval development (Kruger *et al.*, 1998). Production of large yolk−rich oocytes is costly, leading to the suggestion that Alcyonacea might require longer periods of oogenesis than do hard corals (e.g. Gutiérrez−Rodríguez and Lasker, 2004). However, large oocytes are apparently found in octocorals regardless of the length of oogenesis (Kruger *et al.*, 1998) and there is no clear correlation between these traits (Benayahu and Loya, 1986). Oogenesis might be as variable as 10 − 24 months (Benayahu and Loya, 1986; Gutiérrez−Rodríguez and Lasker, 2004 and others). Studies on tropical broadcast spawning alcyonacean corals described spermatogenesis as an annual event, whereas oogenic cycles overlap, the production of the next generation of oocytes starting before the spawning event (Yamazato *et al.*, 1981; Alino and Coll, 1989; Benayahu, 1997a). For the commercially important precious corals, *Paracorallium japonicum, Corallium elatius,* and *C. konojoi* in Japan, spermatogenesis was described to occur annually whereas oocytes were produced over a biennial cycle (Nonaka *et al.*, 2015). With the exception of July, sperm sacs were found all year round in all three species, suggesting that spawning occurred between June and July. Although in Japan the precious corals occur in deep water and do not have photosynthetic symbionts, they are exclusively broadcast spawners rather than brooders.

Some soft coral species display polyp dimorphism − in these species, autozooid polyps have 8 well developed tentacles, whereas siphonozooid polyps have reduced tentacles or none (Bayer *et al.*, 1983). In the Antarctic octocoral *Thouarella variabilis,* a single larva may occupy up to 80 % of the polyp cavity (Brito et al., 1997, as cited in Simpson, 2009), therefore gamete differentiation within the siphonozooids might be crucial adaptation for the survival of the asymbiotic dimorphic octocorals (Simpson, 2009). The author hence concluded that gametes occur mainly in autozooid polyps in shallow−water symbiotic species, which can survive autotrophically, whereas gametes differentiate in the siphonozooid polyps in deep−sea corals.

In contrast to previous suggestions, the gametes develop within the siphonozooid rather than the autozooid polyps in the species of Japanese precious corals listed above (Nonaka *et al.*, 2015).

1.3 Early development

Amongst anthozoans, early development has been described most comprehensively for the hard coral *Acropora millepora* and the starlet sea anemone *Nematostella vectensis,* both of which belong to the subclass Hexacorallia. In these organisms, descriptive embryology has to some extent been complemented by molecular studies (e.g. Anctil *et al.*, 2007; Fritzenwanker *et al.*, 2007; Grasso *et al.*, 2008; Shinzato *et al.*, 2008). Despite the increasing interest in octocorals due to their chemical properties, surprisingly little is known about early development. There is a limited literature on the morphology of early soft coral development (Wilson, 1883; Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998), but later developmental processes, such as infection with symbiotic algae, settlement and survival, have received more attention (Benayahu and Loya, 1987; Benayahu *et al.*, 1988; Benayahu *et al.*, 1989; Benayahu, 1991; Benayahu *et al.*, 1992; Lasker and Kim, 1996; Zaslow and Benayahu, 1996; Kruger *et al.*, 1998; Harii and Kayanne, 2003).

1.3.1 Embryogenesis

Morphological descriptions of early soft coral development have revealed a variety of cleavage patterns (Wilson, 1883; Matthews, 1917). The first cytosolic division does not always result in a 2 cell stage, but predominantly occurs after several rounds of nuclear division, resulting in the appearance of 8 – 32 cell stage (Wilson, 1883; Matthews, 1917; Dahan and Benayahu, 1998). Successive rounds of cell division lead to the development of a spherical embryo and further into a pear shaped planula (Wilson, 1883; Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). In contrast to hexacorals, in octocorals there are no morphologically characteristic stages between the blastula and the planula, so the timing of gastrulation is unknown. Octocoral embryos are thought to gastrulate predominantly by delamination, *i.e.* one cell layer splits or migrates off to form two cell sheets (Wilson, 1883; Matthews, 1917).

In the hexacorallians, *Nematostella vectensis* and *Acropora millepora* gastrulation occurs by invagination (Babcock and Heyward, 1986; Ball *et al.*, 2002b; Fritzenwanker *et al.*, 2004; Kraus and Technau, 2006). Morphologically the process of gastrulation can be determined in *A. millepora* when embryos develop from the concave–convex dish shaped 'prawn chip' stage into the 'fat donut' (Hayashibara *et al.*, 1997; Miller *et al.*, 2000) (Figure 1.2). Even though *N. vectensis* embryos go through stages of slightly concave–convex shapes,

they do not develop into a 'prawn chip' such as in *A. millepora,* and gastrulate from a spherical blastula (Fritzenwanker *et al.*, 2007; Magie *et al.*, 2007).

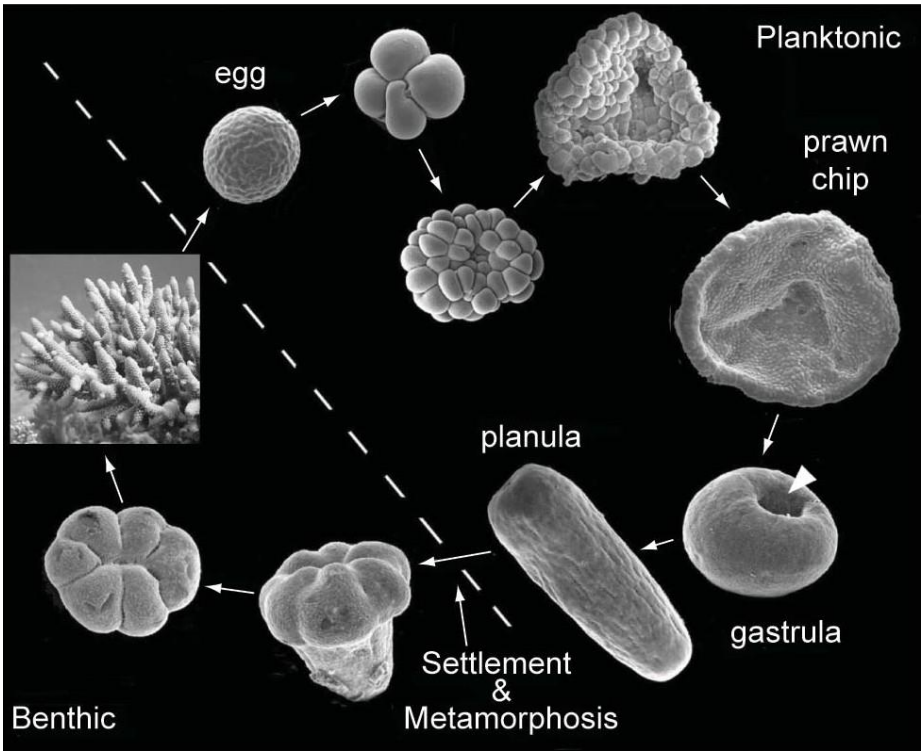


Figure 1.2 The life-cycle of the scleractinian coral *Acropora millepora*. After fertilisation eggs quickly get fertilised in the water column and after successive rounds of cell division lead to the development into the prawn chip stage in about 12 h. The prawn chip then rounds up to form the gastrula (white arrow head indicates blastopore), which then develops into a sphere shaped embryo. The sphere elongates gradually over the following days and eventually reaches competency to settle forming a crown-shaped primary polyp (Hayward et al., 2011).

Post–gastrulation development leads to a planula stage in both *N. vectensis* and *A. millepora*, which metamorphoses into a primary polyp 5 – 10 days and 4 – 7 days post–fertilisation, respectively (Babcock and Heyward, 1986; Kraus and Technau, 2006). Close investigations of the gastrulation process in *N. vectensis* enabled the identification of organising activity in the blastopore lip similar to processes in vertebrates (Kraus *et al.*, 2007). Expression of homologous genes known to play important roles in bilaterian gastrulation, in particular secreted factors, in this blastoporal region (Matus *et al.*, 2006; Rentzsch *et al.*, 2006) implies a common origin of the cnidarian and bilaterian blastopores (Kraus *et al.*, 2007). Comparing localisation of gene expression of key organiser genes between *N. vectensis* and *A. millepora* revealed that expression patterns were generally similar (Hayward *et al.*, 2015). However, differences between the two hexacorallian species allow new interpretations of the role and function of specific genes in the respective species (Hayward *et al.*, 2015). In *A. millepora, brachyury* is the key gene in the demarcation of early endoderm (Hayward *et al.*,

2015), whereas in *N. vectensis forkhead* and *brachyury* are co−expressed around the blastopore, hence their roles are less clear (Fritzenwanker *et al.*, 2004; Hayward *et al.*, 2015). Even within the Scleractinia, conserved homologs showed varying expression patterns from those seen in *A. millepora* and *N. vectensis* (Okubo *et al.*, 2016). The fact that homologous genes are deployed differently between Hexacorallia indicates that we are still far from understanding cnidarian development and the evolution of this process. In order to get a broader view on modes of gastrulation and the genes involved in Anthozoa, comparative data from Octocorallia are crucial for future comparative studies.

In Octocorallia, gastrulation leads to the development of a sparsely flagellated, pear−shaped early planula (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). The mature planula is slender with a broad oral end and a tapered aboral end. The surface is densely flagellated and allows active swimming behaviour in the water column (Matthews, 1917; Benayahu and Loya, 1986; Uehara *et al.*, 1987; Benayahu *et al.*, 1988; Dahan and Benayahu, 1998). In the azooxanthellate octocoral *Dendronephthya hemprichi* from the Red Sea, competency to settle is reached as soon as the mature planula stage is reached (Dahan and Benayahu, 1998). In most octocoral species, however, the development of competence requires a longer period in the water column (Matthews, 1917; Uehara *et al.*, 1987; Benayahu, 1989). The period of competency varies between species, but generally planulae are competent to settle at least for a few days (Benayahu and Loya, 1986; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). In the case of *D. hemprichi*, settlement is asynchronous − planulae settled over a period of up to 2 days (Dahan and Benayahu, 1998). During settlement, competent planulae attach to the substrate with the tapered end (Uehara *et al.*, 1987), become progressively shorter and metamorphose (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Within the first day of settlement, planulae develop into primary polyps with 8 tentacular buds (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Tentacular buds of primary polyps continue to grow and develop into moveable tentacles, which eventually also become pinnate (Matthews, 1917; Uehara *et al.*, 1987).

1.3.2 Metamorphosis and calcification

After settlement and metamorphosis into a primary polyp, the next crucial step in hard coral development is the formation of the exoskeleton. Metamorphosis and onset of calcification are possibly closely correlated. In *Pocillopora damicornis*, for example, metamorphosis is tightly linked to settlement and a few hours after settlement the larval epidermis is replaced by a simple epithelium consisting of only calicoblast cells (Vandermeulen, 1975). Within 6 h post settlement, the first rudimentary skeletal elements are formed, which then fuse to form the larval basal disc within 48 – 72 h (Vandermeulen and Watabe, 1973). Despite intensive research, the process by which this exoskeleton is produced is still under debate (reviewed by Allemand *et al.*, 2004; Allemand *et al.*, 2011). The two contrasting hypotheses are 'biologically induced mineralisation' (Lowenstam, 1981) and 'biologically controlled mineralisation' (Lowenstam, 1981; Mann, 1983). The consensus view is that calcification in Scleractinia is induced extracellularly (Johnston, 1980; Allemand *et al.*, 2011). The presence of intracellular vesicles containing calcium and organic matrix within calicoblastic cells has led to the assumption that exocytosis is involved in the secretion of the organic matrix in hard corals (reviewed in Allemand *et al.*, 2011). The current hypothesis is that calcification is a two−step process in Scleractinia (Cuif and Dauphin, 2005; Cuif *et al.*, 2008). The calicoblastic cells are thought to first secrete nanosized organic cortex, after which calcium ions or amorphous calcium carbonate are believed to enrich this 'nanocortex' (Allemand *et al.*, 2011). Finally, calcium ions crystallise with carbonate ions to form aragonite by a series of chemical reactions (Allemand *et al.*, 2011).

Galaxin, isolated from adult *Galaxea fascicularis,* was the first protein component of the coral organic matrix to be identified (Fukuda *et al.*, 2003). This protein is characterised by a tandem repeat structure and the occurrence of dicysteine repeats, highlighting the possibility for crosslinking of this protein for the formation of a macromolecular network (Fukuda *et al.*, 2003). In *A. millepora*, 3 transcripts related to Galaxin containing dicysteine−rich repeats were identified and predicted to be extracellular (Reyes−Bermudez *et al.*, 2009). One of the three transcripts was a clear ortholog of the *Galaxea* protein, whereas the other two encoded larger and more divergent proteins. While the two Galaxin−like transcripts of *A. millepora* are exclusively expressed during the onset of calcification, the Galaxin ortholog is expressed only in post−settlement and adult stages (Reyes−Bermudez *et al.*, 2009). The expression pattern of the latter is consistent with that of its *G. fascicularis* ortholog, and suggests a role of this protein in the adult skeleton (Fukuda *et al.*, 2003; Reyes−Bermudez *et al.*, 2009). Carbonic anhydrases (CAs) located in the calicoblastic epithelium in scleractinian corals, are another

important group of proteins in calcification (Hayes and Goreau, 1977). CAs favour the hydration of carbon dioxide and are therefore believed to play important roles in the increase of carbon dioxide hydration in the nanocortex (Allemand *et al.*, 2011). Proteome sequencing of the *A. millepora* organic matrix identified 36 skeletal organic matrix proteins (SOMPs), including secreted proteins such as Galaxin and extracellular regions of transmembrane proteins, leading to the proposal of a close control of the skeleton deposition by the calicoblastic epithelium (Ramos−Silva *et al.*, 2013). This study highlighted that the organic matrix consists of not only acidic proteins but rather a wide range of proteins with a large spectrum of potential functions and that not all SOMPs are secreted but some are membrane−bound. A proteome study of the organic matrix of *Stylophora pistillata* identified some of the same proteins, such as cadherins, collagens, acid−rich proteins, but also others, including ubiquitous cellular proteins such as tubulins (Drake *et al.*, 2013). However Ramos−Silva and colleagues (2013) point out that the identification of tubulins in the matrix by Drake and colleagues (2013) was likely due to contamination with remaining tissue. The family of small cysteine−rich proteins (SCRiPs) identified as lineage−specific gene expansion in hard corals (Sunagawa *et al.*, 2009) were originally thought to function in calcification (Grasso *et al.*, 2008; Sunagawa *et al.*, 2009; Moya *et al.*, 2012). Despite the identification of wide range of proteins identified in the organic matrix and the calicoblastic epithelium, their role in coral calcification is yet to be determined (Allemand *et al.*, 2011). Different expression patterns of transcripts within each group of proteins made assignments of definite roles difficult so far. Exposing *A. millepora* primary polyps to high CO_2 conditions for example gave contrasting results (Moya *et al.*, 2012). Whereas some adult−type galaxins and SCRIPs showed increased expression, other proteins of both families and more coral−specific genes were down regulated. This study revealed an unbiased perspective of acidification on early stages of scleractinian calcification and highlighted the complex interactions of these proteins (Moya *et al.*, 2012).

In octocorals the timing of the onset of calcification after settlement and metamorphosis is variable between species. Matthews (1917) observed sclerite formation in the polyp body of *Alcyonium digitatum* on the second day after metamorphosis. This process took considerably longer in *Dendronephthya hemprichi* and *Lobophytum crassum*, 3 − 4 days and up to 2 weeks, respectively (Uehara *et al.*, 1987; Dahan and Benayahu, 1998). For gorgonian octocorals, calcification is described as initially intracellular, followed by an extracellular phase (Goldberg and Benayahu, 1987; Grillo *et al.*, 1993). In the intracellular step, electron lucent vesicles are formed within the scleroblasts. These vesicles then calcify and grow before forming one or more intracellular masses of polymorphic crystals, which are

released to the mesoglea (Goldberg and Benayahu, 1987). In the extracellular space, the mesoglea, secondary scleroblasts attach to these primordial sclerites and produce layers of calcite crystals with irregular protuberances (Goldberg and Benayahu, 1987; Grillo *et al.*, 1993). As a result, several primordia are integrated into a single immature sclerite, which continues to grow by calcite deposition of secondary scleroblasts (Goldberg and Benayahu, 1987). This process was also confirmed for alcyonarian octocorals by Jeng and colleagues (2011). At the molecular level, extracellular proteins have been identified that can induce calcite formation in vitro (Rahman *et al.*, 2011). Further organic matrix proteins including carbonic anhydrases, an actin isoform and a previously unidentified protein 'scleritin', were identified and suggested to play vital roles in soft coral calcification (Lucas and Knapp, 1996; Rahman *et al.*, 2007; Debreuil *et al.*, 2012; Rahman *et al.*, 2013).

Comparative molecular analyses highlight ancestral roles in several gene families by uncovering similarities in orthologous genes between higher animals and Cnidaria (e.g. Miller *et al.*, 2000; Hayward *et al.*, 2004; Okubo *et al.*, 2016). These studies have also revealed highly diverse and distinct patterns from bilaterian orthologs (Knack *et al.*, 2008) and different expression patterns of orthologous genes between *A. millepora* and *N. vectensis* (Shinzato *et al.*, 2008). These results are in line with developmental differences between the limited range of organisms that have been studied to date, and clearly indicate the need for comparative data from other cnidarians to clarify the inference of the ancestral characteristics (Shinzato *et al.*, 2008). To draw conclusions on general patterns of reproductive traits, larval behaviour and life histories and to gain insights into the diversity of cnidarian developmental strategies, octocoral molecular data are clearly needed.

1.4 Microbiome

The term microbiome describes 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that share the body space' (Lederberg and Mccray, 2001). Scleractinian corals probably benefit from the microbial associates in terms of nitrogen fixation (Rohwer *et al.*, 2002; Lesser *et al.*, 2004) and antibiotic production (Ritchie, 2006). Within hard corals, studies have identified highly diverse though often conserved microbial populations associated with coral species (Rohwer and Kelley, 2004; Bourne *et al.*, 2008).

1.4.1 Microbial community and its function

When talking about microbial communities, one has to keep in mind that all multicellular organisms should be considered holobiont or metaorganism, which is a complex community of many species, the macroscopic host and its associated prokaryotic and eukaryotic species (Figure 1.3) (Bell, 1998; Turnbaugh *et al.*, 2007; Bosch and McFall−Ngai, 2011). The function of a microbial community cannot be described without taking the holobiont approach into account.

In reef ecosystems, the vast majority of microbial studies have been conducted on Scleractinia. The microbial communities associated with hard corals are highly diverse but to some extent species specific (Rohwer *et al.*, 2002; Rohwer and Kelley, 2004). A conserved microbial population with beneficial effects such as antimicrobial activity was found to be maintained in the studied species (Rohwer and Kelley, 2004; Ritchie, 2006; Bourne *et al.*, 2008). Prokaryotes commonly associated with Scleractinia include representatives of the Proteobacteria, Actinobacteria, Flavobacteria, Planctomyecetes, Cyanobacteria and Spirochaetes (Vega Thurber *et al.*, 2009; Littman *et al.*, 2011; Lema *et al.*, 2014a, b; Ainsworth *et al.*, 2015). Bacteria are commonly found in association with the coral epithelia, but have also been localised in the coral mucus, connected to the coral skeleton and a few may be intracellular. It has been suggested that nitrogen fixing cyanobacteria in the tissue of the Caribbean coral *Montastrea cavernosa* might play a role in nitrogen cycling within the association (Lesser *et al.*, 2004). In many corals, the photosynthetic partner provides an additional niche for microbes. Ainsworth and colleagues (2015) demonstrated that some bacteria (1 of the 7 taxa that comprise the "core microbiome") were specifically localised within the dinoflagellate cell and others in coral cells that contain *Symbiodinium*. It has also been suggested that bacteria resident in the coral mucus might confer a degree of protection

by providing antimicrobial compounds and interfering with quorum signalling between pathogenic microbes (Shnit–Orland and Kushmaro, 2009; Bosch and Miller, 2016a).

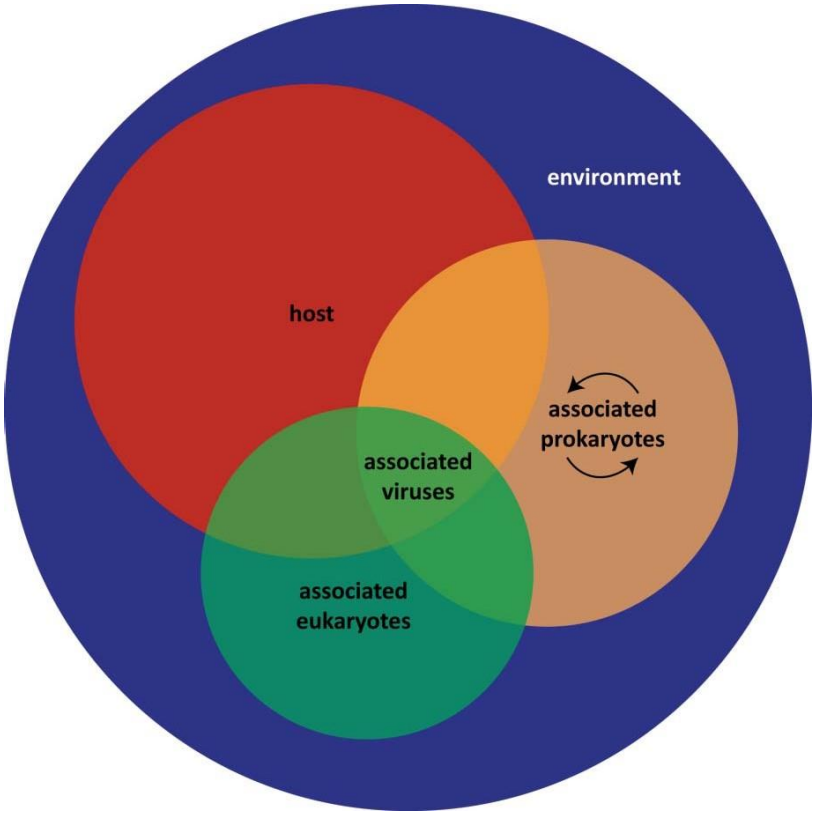


Figure 1.3 All multicellular organisms should be considered as holobiont or metaorganism, as they comprise a complex community of many species, the macroscopic host and its synergistic interactions with the associated prokaryotic and eukaryotic species (Bosch and Miller, 2016b).

Natural variation in the habitat also leads to significant differences between inshore and mid–shelf reefs in the profiles of bacteria associated with corals (Lema *et al.*, 2014b). The microbiomes of corals on inshore reef are typically dominated by y–Proteobacteria, notably members of the *Oceanospirillales* family and of the genus *Endozoicomonas*, whereas a–Proteobacteria were more abundant in corals at mid–shelf reef locations (Lema *et al.*, 2014b). a–Proteobacteria, especially members of the order Rhizobiales, were more dominant during early development and were also present in all stages after settlement in *Acropora millepora* (Lema *et al.*, 2014a). In the case of *Hydra* species, the adult microbiome is established within the first few weeks of development (Franzenburg *et al.*, 2013) and stable throughout time (Fraune and Bosch, 2007). Similarly, the microbial communities associated with early life history stages of *Acropora millepora* are highly diverse and variable but stabilise later on (Lema *et al.*, 2014a). The acquisition or transmission mode of bacteria to coral

embryos may differ between brooding and broadcast spawning corals. Whereas in the case of the brooding coral *Porites asteroides* (Sharp *et al.*, 2012), bacterial communities were found to be similar across developmental stages, suggesting vertical transmission, highly diverse microbial communities between early life history stages and planulae in the broadcast spawning *Pocillopora meandrina* suggests horizontal acquisition of bacteria in this species (Apprill *et al.*, 2012). However the major contribution of *Roseobacter* to the microbial profile of early life history stages in the broadcast spawning corals *A. millepora* and *Pocillopora meandrina* but also in the brooding coral *Porites asteroides* suggests that, irrespective of reproductive mode, some bacteria are likely to be transmitted vertically (Apprill *et al.*, 2012; Sharp *et al.*, 2012; Lema *et al.*, 2014a).

Recently studies have explored the possibility of a coral core microbiome, a subset of microorganisms shared by most individuals within a species, which are assumed to have crucial roles within the association (Ainsworth *et al.*, 2015). These communities are hypothesised to be important to host health during times of stress (Shade and Handelsman, 2012), so a loss of the core microbiome might be expected to severely reduce host fitness. Periods of environmental stress have been shown to drive shifts in the coral microbiome either by a host response andfor changes in the microbiome itself (Bourne *et al.*, 2008). For example, during bleaching events the coral associated microbiomes are more diverse (Bourne *et al.*, 2008), and this can be accompanied by a shift in functional composition from autotrophic to heterotrophic communities (Littman *et al.*, 2011). How the core microbiome is affected by stress may have important consequences for coral health, and is a subject of ongoing investigation.

Few studies have investigated octocoral microbiomes, but γ−Proteobacteria dominate the microbiomes associated with gorgonians. The majority of sequences retrieved from 16S rRNA gene profiling studies were identified as affiliated with the genus *Endozoicomonas* (Sunagawa *et al.*, 2010; Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). That this host−microbial association is symbiotic has been inferred based on its geographic and seasonal stability (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). Further, this association seems to be species−specific, each gorgonian species harbouring a specific *Endozoicomonas* ribotype even on a regional scale, which is consistent with the idea of host−dependant selection (La Rivière *et al.*, 2015).

1.4.2 Stress and immunity

Ever–increasing stresses on coral reef ecosystems, including rising seawater temperatures and ocean acidification, are leading to increases in the frequency, extent and severity of mass bleaching events. Ocean acidification describes the decrease of seawater pH due to increased absorption of atmospheric carbon dioxide (CO_2), which leads to a rising partial pressure of CO_2 (pCO_2) (Kleypas, 2011). During bleaching events, coral associated microbiomes are more diverse (Bourne *et al.*, 2008) and shift from being largely autotrophic to being dominated by heterotrophic species (Littman *et al.*, 2011). In diseased or stressed corals, these communities are dominated by *Vibrio* sp. in yellow band disease (Cervino *et al.*, 2004) and photosynthetic cyanobacteria, sulfate–reducing and sulfide–oxidizing bacteria in black band disease infected corals (Frias–Lopez *et al.*, 2003; Richardson, 2004). γ–Proteobacteria were found in higher abundance in bleached *A. millepora*, while α–Proteobacteria were more abundant in healthy corals (Littman *et al.*, 2011). Differentiating between disease–related and environment–related effects on the microbiome is difficult as the observation of disease signs frequently correlates with overall deterioration of the environment (Lesser *et al.*, 2007). In *Porites compressa,* experimental stress induced shifts in the coral–associated microbial community towards a state resembling that associated with diseased corals (Vega Thurber *et al.*, 2009). The correlation of the loss of symbionts with the loss of *Spongiobacter*–related sequences led to the assumption that losing symbionts might be the driving factor in changing microbiota of corals (Bourne *et al.*, 2008). Alongside the loss of symbionts, loss of antibiotic activity in the mucus during prolonged bleaching facilitates the establishment of non–resident bacteria and represents a potential risk for infection and development of disease (Ritchie, 2006). Changes in the microbial community away from normal homeostasis might also trigger an innate immune response in order to fight off potential infections. The immune system may also influence the associated microbial communities of the coral with an increasing number of studies recognising the importance of microbial conitutents within the coral holobiont (Bosch and McFall–Ngai, 2011). The innate immune repertoire of corals is surprisingly complex and vertebrate like (Lesser *et al.*, 2007; Miller *et al.*, 2007; Shinzato *et al.*, 2011; Palmer *et al.*, 2012). Among those shared components are pattern recognition molecules and signal transduction pathways (Lesser *et al.*, 2007; Miller *et al.*, 2007; Augustin *et al.*, 2010; Shinzato *et al.*, 2011; Palmer *et al.*, 2012; Hamada *et al.*, 2013). Upon exposure to a common fungal parasite, the immune system of the Caribbean sea fan *Gorgonia ventalina* responds with differential expression of pattern recognition molecules and antimicrobial peptides (Burge *et al.*, 2013).

Environmental stress not only affects the microbial community but also the coral holobiont as an entity (Figure 1.3). In the case of a natural high pCO_2 (444 – 953 ppm) site (caused by seepage of volcanic gasses) in Papua New Guinea, decreased calcification rates, reduced coral cover and lower diversity of both hard and soft corals have been observed (Fabricius et al., 2011). The recruitment success of both hard and soft corals was also reduced at high pCO_2 sites relative to low pCO_2 sites (Fabricius et al., 2011). In addition, exposure to sub–lethal temperature stress can impact reproduction in both hard and soft corals. Severe bleaching inhibited gametogenesis in *Montastrea annularis* (Szmant and Gassman, 1990) and fertilization of *Lobophytum compactum* oocytes (Michalek–Wagner and Willis, 2001a). Besides compromising reproductive output, bleaching led to decreased UV protection and weakened oxidative stress defences in *Lobophytum compactum* adult colonies and eggs, threatening egg and larval viability (Michalek–Wagner and Willis, 2001b).

1.5 The Finger Leather Coral *Lobophytum pauciflorum*

The soft coral *Lobophytum pauciflorum* (Ehrenberg, 1834) is common in the shallow tropical waters in the Indo−West Pacific (Tursch and Tursch, 1982; Verseveldt, 1983; Benayahu, 2002) (Figure 1.4). Like most soft corals (Kahng *et al.*, 2011), *L. pauciflorum* is a gonochoric species with dimorphic polyps. The few published accounts of spawning suggest that *L. pauciflorum* is a broadcast spawner, releasing gametes four to five nights after the full moon in early Australian summer on the GBR (Alino and Coll, 1989). *Lobophytum pauciflorum* was observed to spawn during late summer and early autumn in Taiwan when environmental conditions such as water temperature and potential settlement substrate were most favourable (Fan *et al.*, 2005). The gametogenic cycle of this Taiwanese population was found to be around 12 months for both sexes, oogenic cycles therefore potentially overlapping (Fan *et al.*, 2005). Early studies on the GBR showed that terpenoids derived from this soft coral significantly inhibit algal growth and may therefore contribute to the lack of epizoic organisms on the colonies (Coll *et al.*, 1987). Further studies on *L. pauciflorum* have focused largely on the identification and antimicrobial activities of secondary metabolites (Yan *et al.*, 2010a; Yan *et al.*, 2010b; Yan *et al.*, 2011).

Although in the case of *L. pauciflorum*, the possibility of differences in chemical composition between sexes has not been investigated, in the congenic soft coral species *L. compactum*, Michalek−Wagner (2001) observed sex−specific differences in concentrations of UV protective mycosporine−like amino acids. Differences in complementary secondary metabolite concentrations were also observed between female and male colonies of *Sarcophyton glaucum* (Fleury *et al.*, 2006), suggesting the possibility of physiological differences between the sexes. Secondary metabolites isolated from *Lobophytum sp.* were found to have cytotoxic and antimicrobial properties (Zhao *et al.*, 2013), but physiological differences in the properties of secondary metabolites between the two sexes were not investigated in this study. The possibility of chemical and physiological differences between the sexes in soft corals is interesting because sex−specific differences in the expression of immune system components have been identified in bilaterians (Klein, 2000; Peng *et al.*, 2005; Love *et al.*, 2008; Nunn *et al.*, 2009), potentially leading to sex−specific suites of associated microbes.

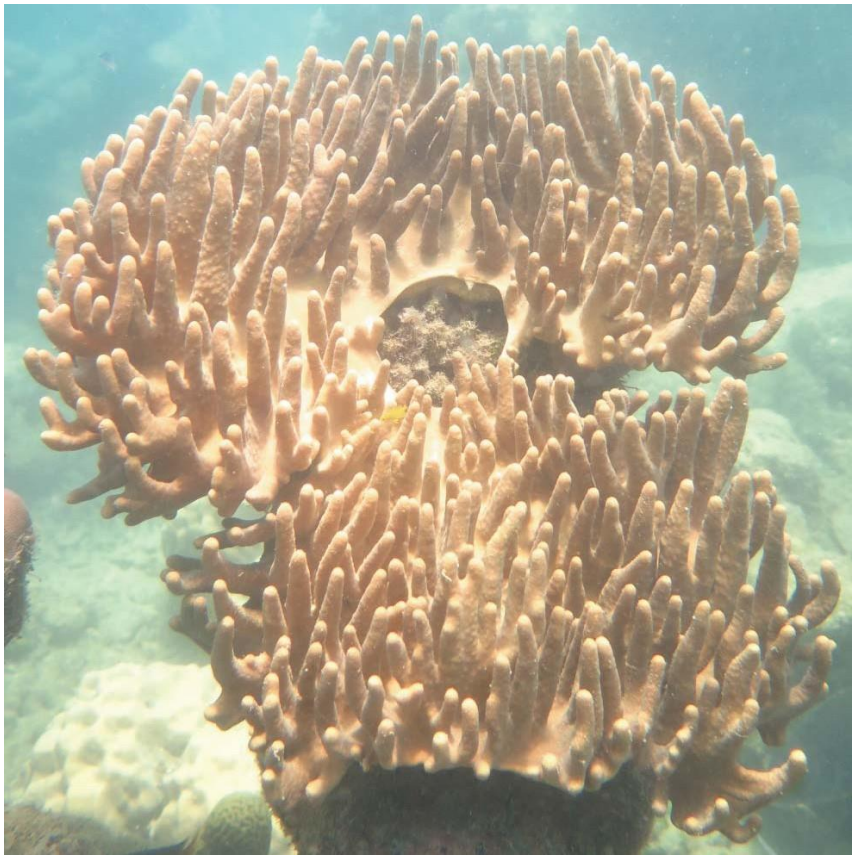


Figure 1.4 Specimen of *Lobophytum pauciflorum* (Family: Alcyoniidae), photographed at Orpheus Island.

1.6 Aims of this thesis

Molecular studies on soft corals are scarce and the early development of alcyonaceans has only been studied at the morphological level. The finger leather coral *Lobophytum pauciflorum* is commoly found in shallow waters on reefs in the Indo−Pacific. The present study aimed to understand the reproductive cycle of *L. pauciflorum* on the Great Barrier Reef compared to two scleractinian corals. Following the identification of reproductive behaviour and development of a method to collect gametes, development of *L. pauciflorum* was investigated at the morphological and molecular level. Taking advantage of the gonochoric nature of *Lobophytum*, the microbial community was characteristed, testing for differences between sexes and assessing the impact of environmental stressors.

Specific objectives and questions addressed in this thesis were:

1. To document gametogenesis and different reproductive strategies in broadcast spawning corals.
    a. What are the patterns of sexual reproduction and sex allocation in *Fungia concinna?*
    b. Is oogenesis in gonochoric scleractinian corals more similar to oogenesis in gonochoric alcyonaceans or more similar to scleractinian corals?
2. To establish the morphological and molecular bases of development in soft corals.
    a. Are the morphological characteristics of soft coral development similar to those known in *Acropora millepora*?
    b. Are the genes involved in early development similar to those in scleractinian corals?
3. To analyse the soft coral core microbiome, to test for sex specific differences and assess the impact of environmental stress.
    a. What are the characteristics of the microbiomes of female and male *Lobophytum pauciflorum*?
    b. What are the impacts of environmental stressors on the soft coral microbial community?

Chapter 2 A comparative study on sexual reproduction of scleractinian and alcyonacean corals

This chapter is inserted with abstract as for submission to Coral Reefs.

Wessels W, Sprungala S, Ball E, Miller DJ (to be submitted to Coral Reefs) A comparative study on sexual reproduction of scleractinian and alcyonacean corals.

Collection of data sexual reproduction and sex allocation in *Fungia concinna* was coordinated by S. Sprungala, data for gametogenesis were collected and analysed by W. Wessels. W. Wessels wrote the chapter and manuscript after intellectual contributions by all co–authors.

2.1 ABSTRACT

Coral reproduction has been a major focus of research for the 30 years since the discovery of mass coral spawning at the Great Barrier Reef and at other locations. Corals involved in these events are mainly broadcast spawners, releasing gametes into the water column where fertilisation and early development occurs until the larvae settle and metamorphose into polyps. The majority of Scleractinia are simultaneous hermaphrodites. However, sequential hermaphrodites, i.e. corals with the potential for sex change, were described recently. In the first part of this study, we report both protandrous sequential hermaphroditism and bidirectional sex change in *Fungia concinna*. This is the second time that bidirectional sex change has been observed in a fungiid species. Secondly, we studied the process of gametogenesis in three coral species, the simultaneous hermaphrodite hard coral *Acropora millepora*, the sequential hermaphrodite scleractinian *F. concinna* and the gonochoric soft coral *Lobophytum pauciflorum*. All three corals reproduced during the mass−spawning event in early summer on the Great Barrier Reef, although *L. pauciflorum* was found to spawn a lunar month ahead of the hard corals in favourable years. The advanced spawning time of *L. pauciflorum* might be an advantage in competition with congeneric species for suitable settlement substrate.

As oogenesis and spermatogenesis started earlier in the soft coral than in the hard corals, the duration of gametogenesis was more similar in *A. millepora* and *F. concinna* than between the gonochorically reproducing *F. concinna* and *L. pauciflorum*. Whereas gametogenesis in shallow−water octocorals was previously thought to occur predominantly in autozooid polyps, in the present study gametes occurred primarily in the siphonozooid polyps in *Lobophytum*.

2.2 INTRODUCTION

Coral reproduction has been extensively studied for the last 30 years, but until recently research on this topic has been almost exclusively ecological rather than molecular. Two different modes of reproduction are generally recognised in corals: broadcast spawning and brooding. Generally brooding occurs internally in the maternal polyp. In some soft coral species, however, larvae are reared externally, on the surface of the maternal colony (e.g. Benayahu and Loya, 1984; Benayahu *et al.*, 1989). Under normal conditions, broadcast eggs are quickly fertilised, which is in line with the generally rapid development. While the vast majority of scleractinian corals are simultaneous hermaphrodites (Fadlallah, 1983), solitary hard corals such as the Fungiidae are either male or female (Fadlallah, 1983; Krupp, 1983). Solitary corals display a wide variety in reproductive strategies, from year−round brooding of large planulae (Fadlallah and Pearse, 1982a; Tranter *et al.*, 1982) to participation in annual spawning events during which small eggs are released (Fadlallah and Pearse, 1982b). A size related sexual dimorphism, males typically being smaller than females, is also common in solitary scleractinians, indicating that either males undergo sexual maturity earlier or size−related sex changes (from male to female) occur. In contrast to the situation in *Fungia scutaria,* no relationship between size and sex was observed in *F. granulosa* (Kramarsky−Winter and Loya, 1998), therefore conclusions on the ability of Fungiidae to change sex could not be made by the authors. However, male to female sex change has been recorded in *F. repanda* and *Ctenactis echinata,* confirming the hypothesis that some fungiids are sequential hermaphrodites (Loya and Sakai, 2008). The situation in *C. echinata* appears to be even more complex, as bidirectional sex changes have been observed at intermediate sizes (Loya and Sakai, 2008). The later study not only suggests that the ability to change sex increases the overall fitness of dioecious coral species, but also illustrates the extreme reproductive plasticity of the Scleractinia, which is one reason for their evolutionary success (Loya and Sakai, 2008).

Male to female sex change during life is known as protandry and observed in a range of higher animals, including clown fish and barramundi, as well as corals. Protogynous sequential hermaphrodism (female to male sex change) is also common in marine fish, examples being wrasses and groupers. Changes to a reproductively more successful sex are often size or age related (Ghiselin, 1969; Warner, 1975; Charnov, 1982; Policansky, 1982; Warner, 1988). The advantage of switching from male to female, or vice versa, is related to maximising the odds of successful reproduction and numbers of offspring. A benefit of protandrous sex change is that frequently female fecundity correlates with body size, as

oogenesis is an energetically costly process. On the other hand, larger male body size may be an advantage in terms of the ability to defend a territory or other resources that enable successful reproduction. Loya and Sakai (2008) postulate that bidirectional sex change in *C. echinata* could be a flexible response to changes in environmental conditions. Hence becoming male again could be an adjustment to injuries, disease or shading as resources to gamete production can be partitioned.

The gametogenic cycle for hard corals is species specific but is generally completed in less than one year. The duration of oogenic cycles is more variable than spermatogenic cycles, ranges being 3 − 11 months for the former and 6 − 12 weeks for the latter (Kojis, 1986; Szmant, 1986; Kramarsky−Winter and Loya, 1998). For *Fungia granulosa* and *F. scutaria* in the northern Red Sea, for example, oogenesis was observed to take 7 − 8 months and spermatogenesis 8 − 12 weeks (Kramarsky−Winter and Loya, 1998). In *Acropora spp.,* oocytes were observed over 9 − 11 months prior to spawning, whereas spermatogenesis is reported to take about 6 − 8 weeks (Kojis, 1986; Szmant, 1986). When comparing reproductive patterns among Caribbean reef corals, Szmant (1986) found no correlation of polyp size and morphology with these reproductive patterns. Moreover, the time of onset and duration of gametogenesis did not differ significantly between gonochoric and hermaphroditic hard corals (Szmant, 1986). Within the Faviidae for example, oogenesis in *Montastrea annularis* took 4 − 5 months, whereas this process took up to 9 months in *Diploria strigosa* (Szmant, 1986). The gonochoric *Montastrea cavernosa* was found to start oogenesis about 10 months before the annual spawning event in the Caribbean, which is a similar time frame to *D. strigosa*. Szmant also reported that the gonochoric hard corals, *Siderastrea siderea* and *Dendrogyra cylindrus*, undergo complete oogenic cycles within 3 months (Szmant, 1986). Despite the large degree of variation in oogenic cycles documented, the duration of spermatogenesis was similar (4 − 8 weeks) in all of these species (Szmant, 1986). Irrespective of the mode of reproduction, hermaphroditism or gonochorism, oogenesis generally precedes spermatogenesis in scleractinian species (Szmant−Froelich *et al.*, 1980; Szmant−Froelich *et al.*, 1985; Kojis, 1986; Szmant, 1986; Kramarsky−Winter and Loya, 1998). In the week after a full moon in late Australian spring, over 130 species spawn synchronously at the Great Barrier Reef (GBR) (Willis *et al.*, 1985) and spawning of more than 30 species occurs within hours on the same night at a single location (Willis *et al.*, 1985; Babcock *et al.*, 1986). Some inshore populations spawn one lunar month before populations of the same coral species on offshore reefs. When the full moon occurs in the first half of the months of October and November, spawning is often split over two months, with some

colonies releasing gametes one lunar month before the remainder of the population (Willis *et al.*, 1985).

Soft corals were found to spawn synchronously with the mass spawning of scleractinian corals (Alino and Coll, 1989). In contrast to the Scleractinia, where hermaphroditism predominates, soft corals are predominantly gonochoric, as is also the case in many solitary hard corals (Kahng *et al.*, 2011). Whereas gametogenesis has been extensively studied in hard corals, this process received limited attention in soft corals until relatively recently (Fan *et al.*, 2005; Yeung and Ang, 2008; Simpson, 2009; Nonaka *et al.*, 2015). As soft corals lack a skeleton, some species have developed dimorphic polyps and assigned the function of structural support to a specific polyp type known as siphonozooids. In species with polyp dimorphism, the siphonozooids lack tentacles, whereas the autozooid ("feeding") polyps have eight well developed tentacles and are responsible for catching food particles from the water column (Bayer *et al.*, 1983).

As in the case of hard corals, alcyonacean corals either broadcast spawn gametes, with external fertilisation and development, or brood planulae externally or internally (Benayahu, 1997a). The limited literature on broadcast spawning in tropical Alcyonaceae describe spermatogenesis as an annual event but oogenic cycles as overlapping, suggesting that the production of the next generation of oocytes starts before the spawning event (Yamazato *et al.*, 1981; Alino and Coll, 1989; Benayahu, 1997a). In the commercially important precious corals, *Paracorallium japonicum, Corallium elatius,* and *Corallium konojoi*, spermatogenesis was described as occurring annually, whereas oocytes were produced over a biennial cycle (Nonaka *et al.*, 2015). In these three species, sperm sacs were present throughout the year except for the month of July, suggesting that spawning occurred between June and July. Thus, although these precious corals inhabit the deep−sea and lack photosynthetic symbionts, they appear to be broadcast spawners and, in contrast to previous suggestions (Simpson, 2009), the gametes are located in the siphonozooid rather than the autozooid polyps in these corals. On the GBR, soft corals were found to spawn in early summer in short, synchronised events (Babcock *et al.*, 1986; Alino and Coll, 1989) similar to those of scleractinian corals (Babcock *et al.*, 1986; Harrison and Wallace, 1990).

The alcyonacean coral *Lobophytum pauciflorum* (Ehrenberg, 1834) is abundant and commonly found on reefs in the Indo−Pacific (Tursch and Tursch, 1982; Verseveldt, 1983; Benayahu, 1997b, 2002). The limited observational data available suggest that *L. pauciflorum* is a gonochoric broadcaster, spawning on the GBR four to five nights after a full moon in the

early Australian summer (Alino and Coll, 1989). In Taiwan, Fan and co–authors observed the spawning of *L. pauciflorum* during late summer and early autumn, when environmental conditions such as water temperature and settlement substrate availability (due to typhoon disturbances) were most favourable (Fan *et al.*, 2005). The gametogenic cycle of this Taiwanese population was found to be 12 months for both sexes.

In the first part of this study, the sequential hermaphrodite *Fungia concinna* was assessed over the course of 4 years for spawning behaviour, sex allocation of individual polyps and potential sex change. For both sexes, the timing of the onset of gametogenesis was investigated, in order to determine when sex change could potentially occur. In addition to these data, the reproductive cycles of the simultaneous hermaphrodite *Acropora millepora* and the gonochoric soft coral *Lobophytum pauciflorum* were studied to enable comparative analyses of gametogenesis not only between hermaphroditic and gonochoric hard corals, but also between gonochoric species of Scleractinia and Alcyonaceae. The reproductive cycle and spawning time of *L. pauciflorum* on the GBR are compared to these patterns in the Taiwanese population to draw more general conclusions on the spawning behaviour of this species for future studies on reproduction.

2.3 MATERIALS & METHODS

2.3.1 Sexual reproduction of Fungia *concinna*

Fungia concinna specimens were sampled from 2011 to 2014 in accordance with the GBRMPA permit G11f34573.1 from several bays around Orpheus Island (18˚34' S; 146˚29' E) 3 − 4 nights after full moon (NAFM). Corals were kept in a 1000 L flow through aquaria with 1 µM filtered seawater. Prior to spawning 5 − 7 NAFM, water level was lowered and corals were isolated in 2 L aquaria to allow sex determination assuring that no water connection existed between aquaria. Sex of each corallum was recorded based on the unique shape of shed gametes. After spawning, corals were tagged, measured and weighed before returning the corals to their original location. The following years corals were retrieved late November or early December depending on the full moon. Due to low retrieval rates in 2012, *F. concinna* specimens were placed in closer proximity to each other, about 1 m apart from each other, at least, at a marked location in Pioneer Bay. Each year, data on sex determination and size and weight were collected as described above. To determine when sex change occurs, monthly samples from selected tagged corals were taken over a period of 12 months in 2014ƒ15.

2.3.2 Sample collection and preparation for gametogenesis

Coral samples, i.e. 2 nubbins per colony of *Acropora millepora*, 2 fragments per specimen of *Fungia concinna*, 1 lobe per colony of *Lobophytum pauciflorum* were collected monthly from Little Pioneer Bay at Orpheus Island (18˚34' S; 146˚29' E) at a depth of 2 − 3 m, using bone cutter, hammer and chisel and scissors, respectively (GBRMPA permits G11f34573.1, G12f35295.1, G13f36196.1). 1 nubbinffragment of *A. millepora* and *F. concinna* was immediately snap frozen on dry ice for later molecular biological studies. The second nubbinffragment was fixed for histological analysis. The collected lobe of *L. pauciflorum* was split in two equal halves using a razor blade. One half was then snap frozen on dry ice for molecular biological studies and the other half fixed for histological analysis. Histological samples were promptly fixed after collection for 24 h in 4 % formaldehyde in HEPES buffered seawater (pH 8.0) and then preserved in 70 % ethanol until processing. The samples were decalcified overnight in 10 % formic acid prior to paraffin embedding. Three sections of each sample (5 µm) were stained in Haematoxylin–Eosin to analyze the stage of gonadal development. Gonads were identified using a 10x magnification of a compound stereomicroscope (Olympus BX40). Only the diameter where nucleus was clearly visible was measured under appropriate magnification using the cellSens software (cellSens standard

v1.8) to ensure that the full size of the oocytes was recorded. Gonads were grouped in different size classes relative to the final size of the oocytes. For *A. millepora* and *L. pauciflorum* the size groups were determined as following: Stage I oocytes (< 50 µm), Stage II oocytes (50 − 99 µm), Stage III oocytes (100 − 199 µm) and Stage 4 oocytes (> 200 µm). *F. concinna* oocytes were significantly smaller than *Acropora* and *Lobophytum* oocytes ($p < 0.05$). The size groups were therefore adjusted for this species: Stage I oocytes (< 25 µm), Stage II oocytes (25 − 49 µm), Stage III oocytes (50 − 74 µm) and Stage 4 oocytes (> 75 µm).

In order to test for possible correlation of oocyte development with water temperature, data for daily average water temperatures at 6.1 m depth for the sampling period at Orpheus Island were downloaded from the AIMS data centre for relay pole 2 (http:ƒƒdata.aims.gov.auƒaimsrtdsƒdatatool.xhtml?site=9).

2.3.3 Statistical analysis

Statistical analysis was undertaken using GraphPad Prism (Version 6). Average oocyte sizes and temperature data were tested for normality (Kolmogorov−Smirnov) and homogeneity of variances (Bartlett). Linear regression and correlation of oocyte size with temperature (Pearson) were calculated from June till November.

2.4 RESULTS

2.4.1 Sexual reproduction and sex allocation in *Fungia concinna*

Fungia concinna were gonochoric (Figure 2.3 B & E), only specimens over 50 mm in length being reproductive (Figure 2.1, Figure 2.2 A). At this small size the majority of specimens (8 of 23 individuals) reproduced as males, however two individuals in the 50 − 75 mm size range (i.e. 2 of 23) and six of between 75 − 100 mm (6 of 34 individuals) reproduced as females (Figure 2.2 A). Overall, however, the majority (17 of 27; 63 %) of specimens collected smaller than 75 mm were non−reproductive (Figure 2.1, Figure 2.2 A). Female reproduction in *F. concinna* appears to require individuals to be at least 100 g. Although some females were lighter (7 of 51), the female to male ratio approached 1 for individuals in the 100 − 200 g range (Figure 2.2 B). Above 125 mm in length and a weight of 300 g, the *F. concinnca,* polyps were predominantly female (Figure 2.2). Specimens larger and heavier than 175 mm and 400 g, respectively, were non−reproductive. At an intermediary size and weight of 125 mm and about 180 g, the sex ratio was approximately equal (Figure 2.1, Figure 2.2). Size and weight ranges for sex change in *F. concinna* were between 100 − 150 mm and 125 − 150 g, respectively (Figure 2.1, Figure 2.2). However, larger males and smaller females were observed. The relationship between length (L) and weight (W) could be described by the following regression $W = 9.13 \times 10^{-3} L^{2.07}$ with a fit of $R^2 = 0.6162$, N=141.

Approximately 60 % of the corals collected in 2011 (14 of 23) and 2012 (19 of 28) reproduced. This number increased slightly to 66 and 70 % in 2013 and 2014 when more tagged corals were retrieved alive (Table 2.1). Over all collected specimens, the sex ratio was somewhat male biased, with 35 reproductive females and 50 reproductive males, 1:1.4. However over the course of this study the sex ratio varied. Whereas there was a male bias in 2011 and 2012 (ratios 1:1.3 and 1:1.4, respectively), there was then a female bias in 2013 (1:0.8). This female bias then disappeared within the following reproductive season and sex ratio was strongly male biased in 2014, 1:3.8 (Table 2.1).

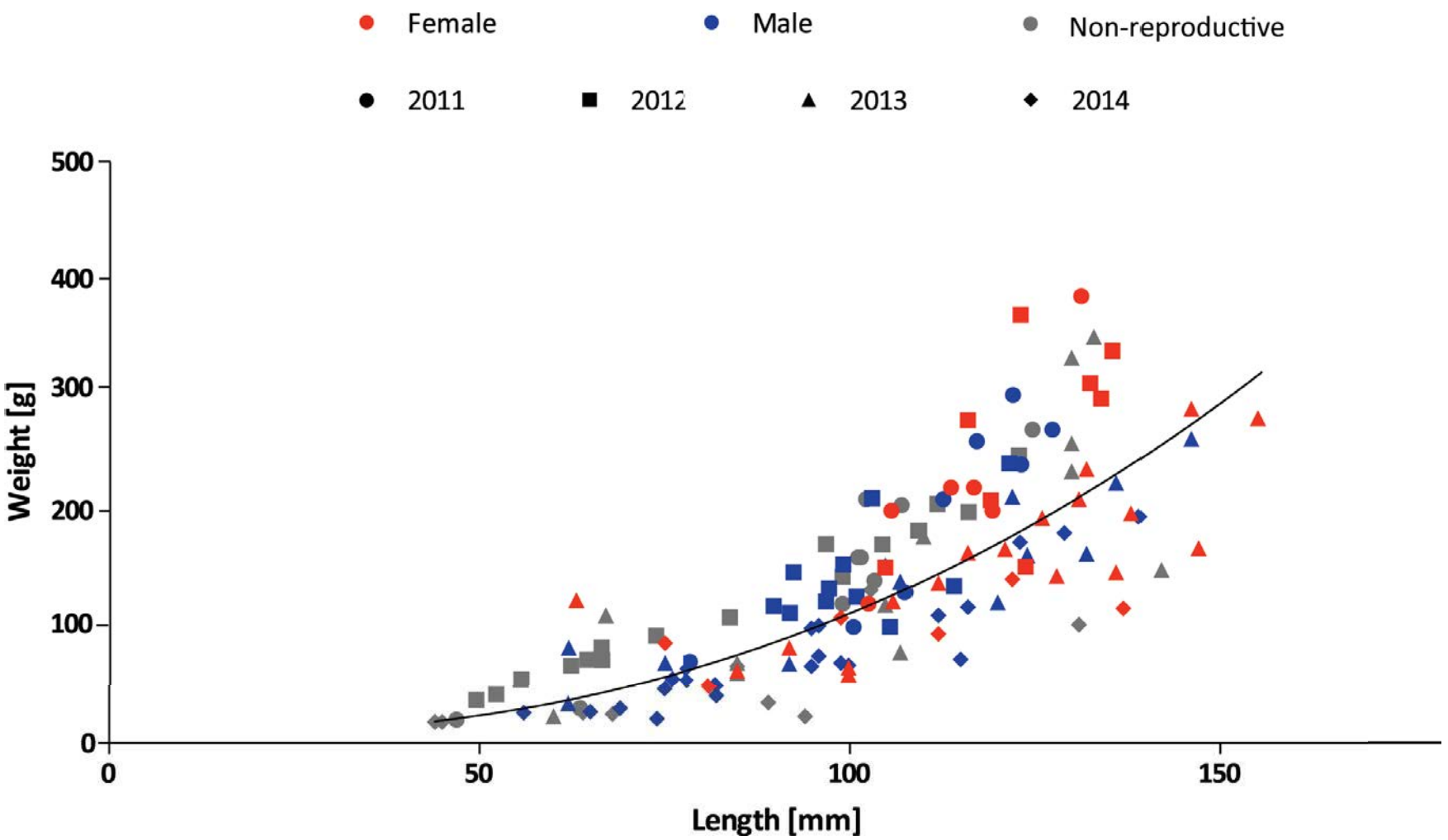


Figure 2.1 Allometric relationship and size range at sex change in *Fungia concinna* showing small non-reproductive specimen (NR, grey symbols) and indicating that sex change was predominantly male to female at smaller size, however female to male sex change was also observed at larger size. Red symbols, females (F); blue symbols, males (M. 2011, circles; 2012, squares; 2013, triangles; 2014, diamonds. N= 141, spread over 38 F, 53 M, 50 NR.

The first sex change in *F. concinna* was observed in 2013 as only one tagged specimen from the first sampling in 2011 was retrieved in 2012 (Table 2.1). However this specimen had reproduced as female in 2011 and 2012, it reproduced as male in 2013. Further that year, 4 specimens previously reproducing as male had changed their sex to female (Table 2.1). In 2014, contributing to the strong male biased sex ratio were 11 individuals that had previously reproduced as female. In three of these cases, however, a sex change (male to female) had previously occurred. In addition, a single individual that had previously undergone female to male sex change in 2013 reverted back to again reproduce as a female in 2014 (Table 2.1).

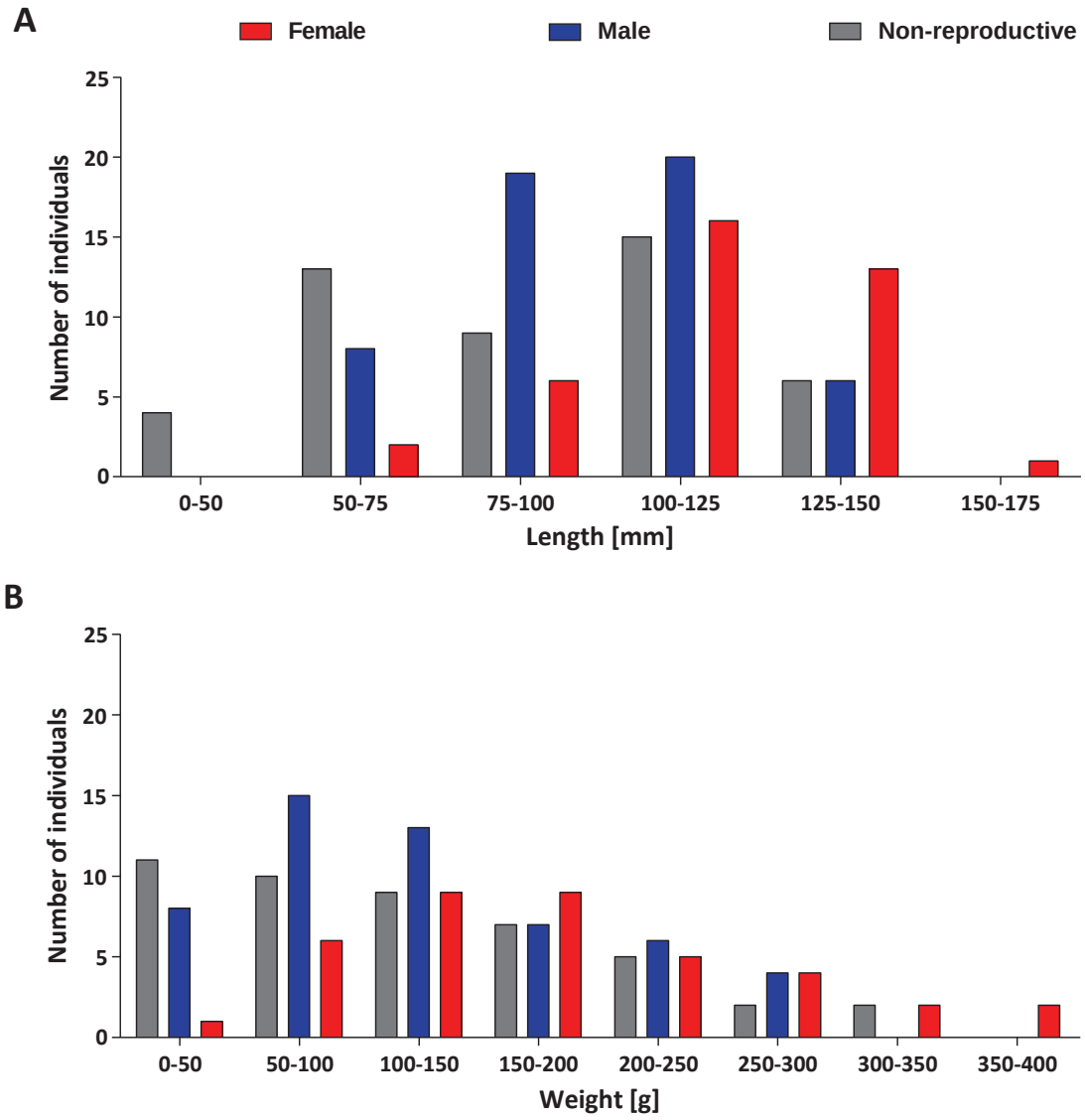


Figure 2.2 Size (A) and weight (B) class distribution of all female (red, n=38), male (blue, n=53) and non-reproductive (grey, n=50) *Fungia concinna* colonies collected over the 4-year study period.

Table 2.1 Recorded population statistics of reproductive strategies and life history traits in sex-changing *Fungia concinna* individuals collected from 2011-2014. Female (F), male (M), non-reproductive (NR), not applicable (n.a.)

	2011	2012	2013	2014
Total corals collected	23	28	39	31
No. of corals retrieved alive	n.a.	1	13	31
Observed breeding	6F, 8M	8F, 11M	16F, 12M	5F, 19M
Non-reproductive (NR) (but reproductive the previous year)	n.a.	n.a.	2F	4F, 3M
Same sex in successive years	n.a.	1F	1F, 1M	2F, 7M
Number of individuals changing sex and direction of sex change	n.a.	n.a.	1F to 1M and 4M to 4F	11F to 11M and 3M to 3F
Number of individuals with bidirectional sex change	n.a.	n.a.	n.a.	1 (F–M–F); 3 (M–F–M)
New individuals added	n.a.	7F, 11M, 9NR	10F, 7M, 9NR	n.a.

2.4.2 Gametogenesis

The observation of both female to male and male to female sex changes in *Fungia concinna* in 2013 led to questions as to the timing of the initiation of gametogenesis, as this effectively limits the time window during which sex change occurs. In order to establish a comparative baseline of gametogenesis in hermaphroditic hard corals but also to allow comparison to gonochoric soft corals, this part of the study was extended to *Acropora millepora* as a representative (simultaneously) hermaphroditic scleractinian and *Lobophytum pauciflorum* as a representative gonochoric alcynacean.

Combining observations of long–term reproductive strategies and with monthly sampling allowed narrowing down the window for sex change between November and February in *Fungia concinna. Fungia* individuals were selected based on their sex during the November spawning in 2013. From Feburary 2014 to February 2015, single male and female individuals were sampled at two monthly intervals; two individuals of each sex were sampled in February 2014, and another two (different individuals) of each sex in the following month, allowing each individual two months to recover after sampling. The targeted male specimen sampled in February had in fact already switched sex and started oogenesis (Figure 2.3 E). The specimen selected the same month and reported as female in November 2013 had also switched sex and reproduced as male during the November spawning 2014. The selected specimens sampled in March 2014 had not changed sex and reproduced as same sex during the November spawning in 2014. The second selected female had likewise to the February specimen already started oogenesis when sampled in March. Oocytes were observed in *F. concinna* every month from February 2014 until late November 2014, but not in material collected in December 2014 and January 2015. At the end of the sampling period, oogenesis had been initiated, as small oocytes were observed in February 2015 samples (Figure 2.6). Note that the number of oocytes varied greatly between months, possibly as a consequence of differences in positions of where the tissue sample was taken on the coral. To minimise damage of the corallum, the sampling position could not be consistent throughout the study period. Spermatogonia were observed for the first time in samples taken in late September, 8 weeks before spawning (Figure 2.3 B). Nuclei of spermatocytes were large and the boundary of the spermary was distinct (Figure 2.3 B).

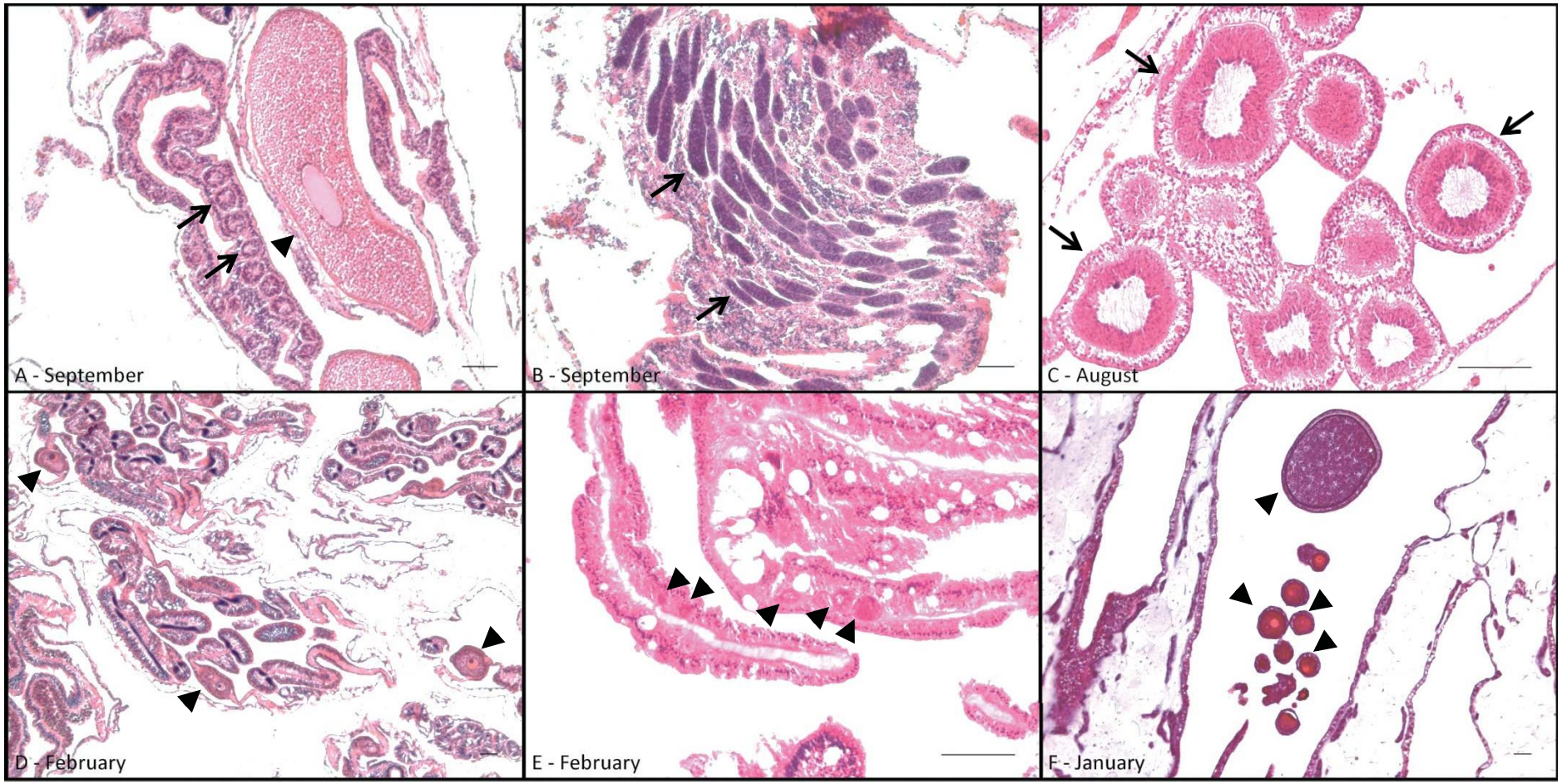


Figure 2.3 Spermatogonia (A – C, arrows) and oocytes (D – F, arrow heads) of *Acropora millepora* (A, D), *Fungia concinna* (B, E) and *Lobophytum pauciflorum* (C, F) the first time they were observed under the microscope in Haematoxylin-Eosin stained sections. Oocytes were observed in *A. millepora* and *F. concinna* 9 months and in *L. pauciflorum* 10 months before spawning, spermatogonia were observed 8 weeks in *A. millepora* and *F. concinna* and 12 weeks in *L. pauciflorum* prior to the spawning event in November. Scale bar indicates 50 µm.

Monthly sampling of five randomly selected colonies of the simultaneous hermaphrodite *A. millepora* started in February 2014. Every month, 5 different colonies were selected for sampling. A few colonies sampled both in February and March likewise had started oogenesis, 1 of 5 colonies in February and 3 of 5 colonies in March (Figure 2.5 A). Oocytes were consistently found in *A. millepora* colonies from April on until late November (Figure 2.5 A, Figure 2.6). As in *F. concinna,* no oocytes were found in histological sections prepared from *A. millepora* samples taken in late December 2014 and January 2015 (Figure **2.5** A, Figure 2.6). Sperm sacs with spermatocytes bearing large nuclei were observed for the first time in late September (Figure 2.3 A). At that time, spermatocytes were arranged peripherally around a prominent central lacuna (Figure 2.3 A).

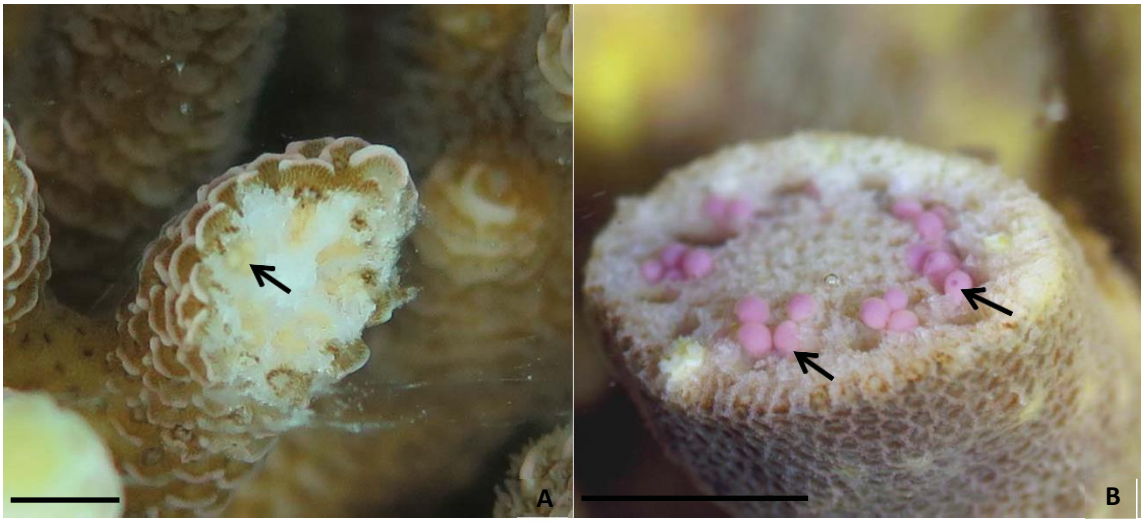


Figure 2.4 Gonad bundles in *Acropora millepora* (A) and individual oocytes in *Lobophytum pauciflorum* (B) in late November and October, respectively. Arrows mark the bundles and oocytes. Scale bars represent 500 µm.

The first sampling for *L. pauciflorum* was conducted in January 2014 using five of the six colony aggregations that were used to observe coral spawning in November 2013. The following month, five different colonies from these aggregations were sampled each month. In January 2014, oocytes of different size groups were observed, 91 % were smaller than 50 µm, 3 % between 50 − 99 µm and 6 % 100 − 199 µm (Figure 2.5 B). Average oocyte size increased over the period February to September. Oocytes larger than 100 µm were not observed in histological samples from February and March (Figure 2.5 B). While taking soft coral samples in October 2014, the oocytes could clearly be observed by the naked eye (Figure 2.4 B). To better understand spawning behaviour, the colonies were carefully monitored over the days immediately after the full moons in September, October and November of 2014, but spawning occurred only on the fourth and fifth eveningfnight after the November full moon, with the

highest activity on the fifth evening. Hence the low number of counts recorded for October might be misleading (Figure 2.5 B). The majority of each *L. pauciflorum* aggregation spawned in November 2014. Only one female colony was found still bearing oocytes at the end of November 2014 but these were not present in December (Figure 2.5 B, Figure 2.6). In *L. pauciflorum*, clusters of spermatocytes were first observed in August 2014. The spermatozoa were already bearing tails and were arranged around the periphery of the spermary (Figure 2.3 C).

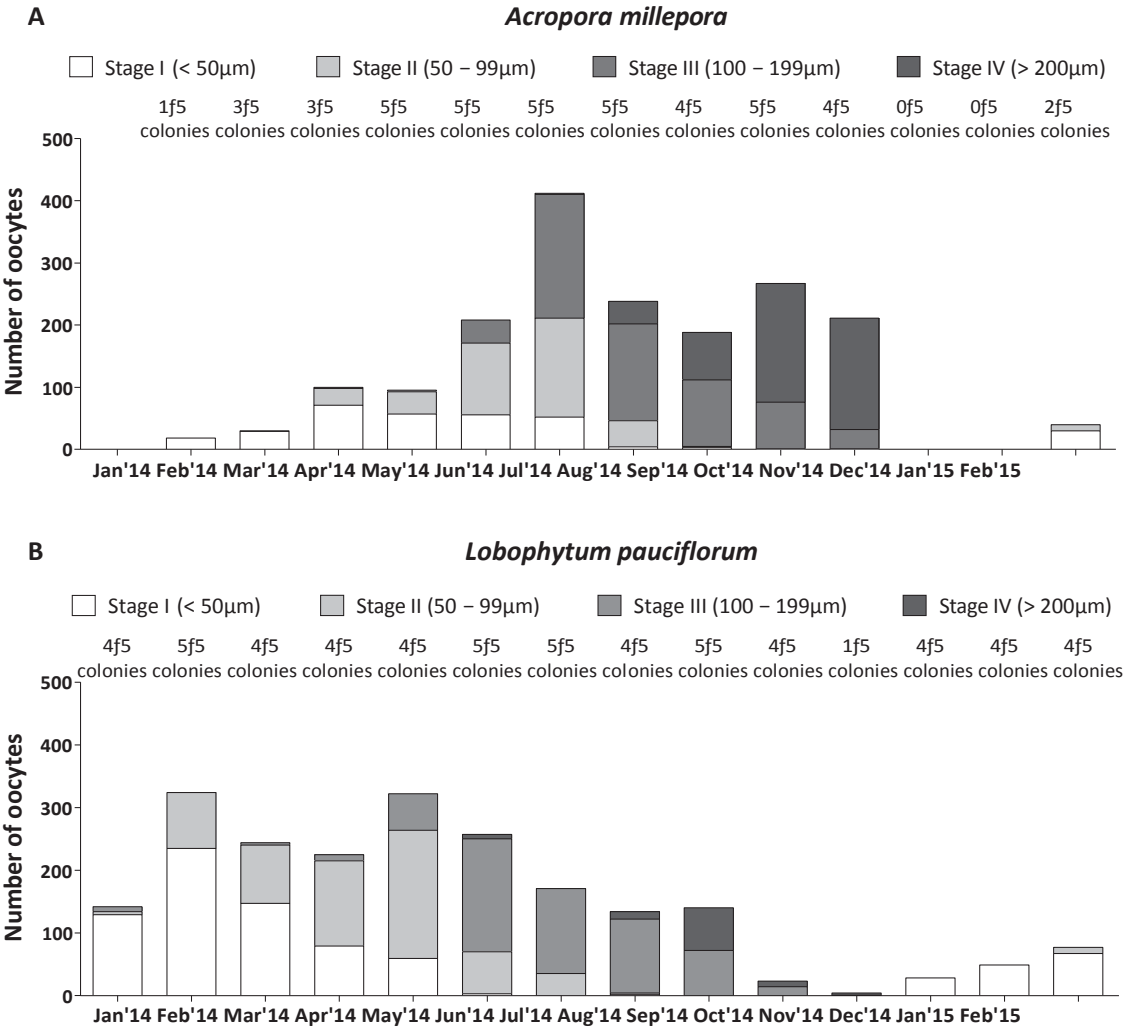


Figure 2.5 Total numbers of oocytes observed in *Acropora millepora* (A) and *Lobophytum pauciflorum* (B) classified in size categories: White bars indicate stage I oocytes (< 50 µm), light grey stage II oocytes (50 – 99 µm), medium grey stage III oocytes (100 – 199 µm) and dark grey stage IV oocytes (> 200 µm). Stage IV oocytes are close to maturation and this size is close to effective spawning size.

In *L. pauciflorum*, oogenesis was observed to commence in January in both 2014 and 2015. Tagged colonies in the field spawned in November of each year. Spermatozoa had already developed tails when observed for the first time in August in the soft coral. Thus, the duration of oogenesis was approximately 10 − 12 months for *L. pauciflorum*, but the duration of spermatogenesis could not be estimated from observations made during this study. While oocytes were not mature in August or September (no spawning occurred), spermatozoa appeared to be fully developed at this time. In *A. millepora* and *F. concinna,* oogenesis started in February. Oogenesis in the two hard coral species took about 9 − 10 months. Spermatogenesis was observed to be in progress in both Scleractinia in September, but to be more advanced in *A. millepora* than in *F. concinna* (Figure 2.3). As for both species spawning was observed in the field after the November full moon in 2014, it is likely that in these cases the duration of spermatogenesis is about 6 − 8 weeks. However, not all gametes were released during the November spawning event, some being retained, possibly for a secondary spawning event in early December (Figure 2.4 A, Figure 2.6). Note that *A. millepora* colonies and *F. concinna* individuals sampled in late December 2014 were depleted of gametes. In contrast to *L. pauciflorum*, oocytes were not found as reliably in *A. millepora* during the early stages of oogenesis (Figure 2.4). Whereas in the case of *L. pauciflorum,* oocytes were found in four of the five colonies sampled from December 2014 to February 2015, only two of the five *A. millepora* colonies were found bearing oocytes in February 2015 (Figure 2.4). In *F. concinna*, oocytes were found in small numbers but reliably in samples in February and March 2014 and in February 2015 (Figure 2.6).

Oogenesis was positively correlated with variation in seawater temperature (Figure 2.6). In late summer and early autumn, while water temperatures were still above 27 ˚C, oogenesis was initiated, but the process appeared to slow from that point, until water temperatures increased again in August. Oocytes increased in size on a linear basis in *A. millepora,* $y = (34.45 \pm 11.15)x - 666.7 \pm 270.5$, and *F. concinna,* $y = (19.84 \pm 4.199)x - 236.8 \pm 171.6$, from June until November. These correlations are significant at p = 0.0088 and p = 0.0022, respectively. For *L. pauciflorum* there is good support (p = 0.0656) for the hypothesis of a linear correlation of average oocyte size and temperature between June and November.

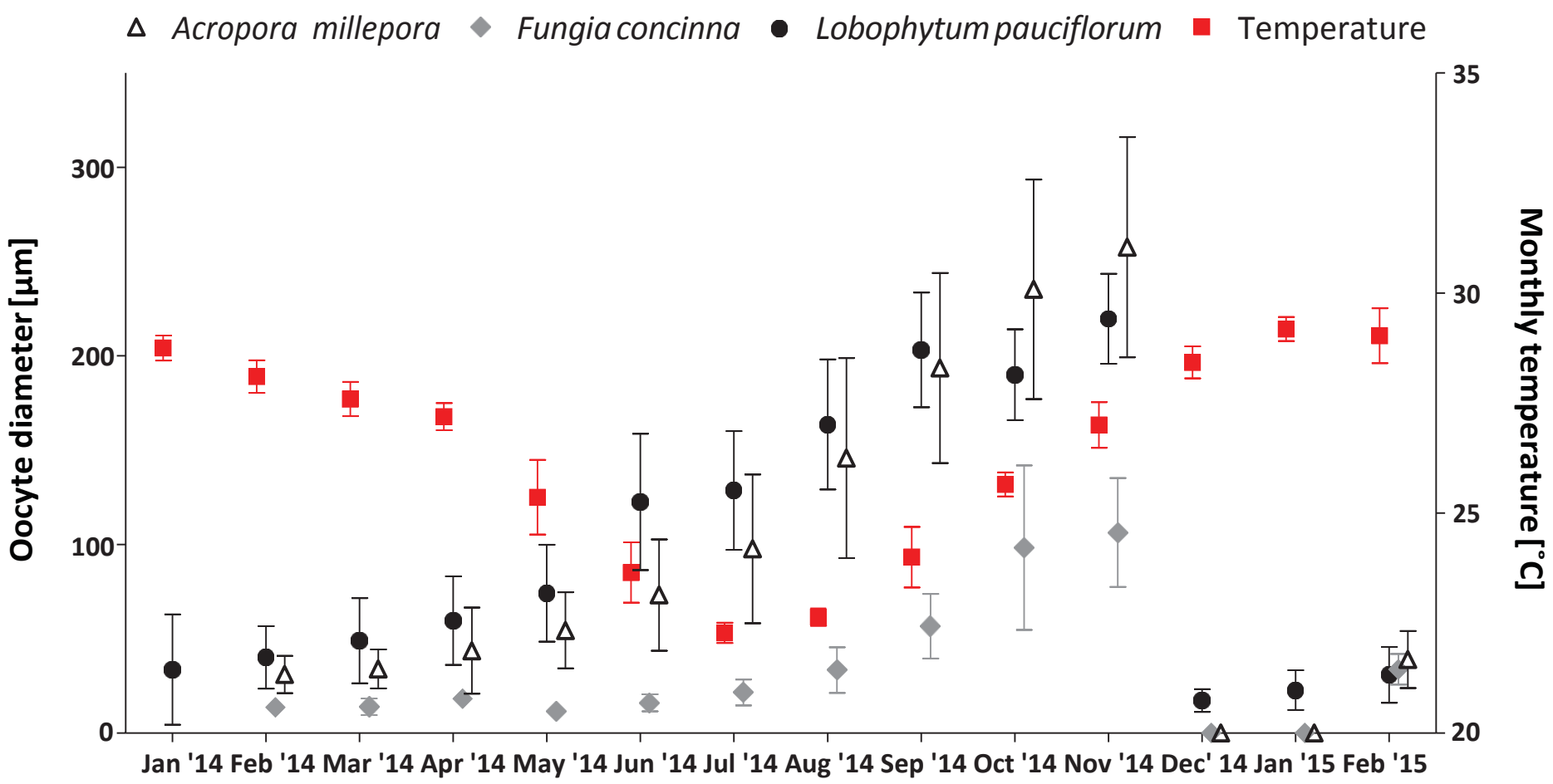


Figure 2.6 Oocyte diameter (with standard deviation) per month for the three coral species investigated, *Acropora millepora* (open triangles), *Fungia concinna* (grey diamonds), *Lobophytum pauciflorum* (black circles) in relation to monthly temperature measured at 6.7 m depth (red squares). Mean oocyte size and mean monthly temperature and standard deviation shown. Spawning of *A. millepora*, *F. concinna* and *L. pauciflorum* was observed in early November.

2.5 DISCUSSION

2.5.1 The potential for bidirectional sex change in *Fungia concinna*

The allometric relationship between size and weight revealed that *F. concinna* individuals at first−sex were lighter (50 − 75 g) than has been documented for *F. repanda* and *C. echinata* (100 − 300 g and 300 − 700 g, respectively)(Loya and Sakai, 2008). The size of first−sex in *F. concinna* recorded in this study 50 − 75 mm (Figure 2.2 A) is similar to what has been reported in *F. granulosa,* where individuals of 55 mm were recorded as males (Kramarsky−Winter and Loya, 1998). These large variations in weight at first−sex display the great sexual plasticity within the family of Fungiidae (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). Data presented here indicate that the relationship between size and sex is not as clear in *F. concinna* as it is in *F. scutaria* or *Ctenactis echinata* (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). It appears rather that, for *F. concinna,* weight is a more important factor in sex determination than is size. Reproductive individuals of less than 100 g were predominantly male (Figure 2.2), and the smallest and lightest reproductive individuals observed in this study were male (Figure 2.1). Life−history traits of *F. concinna* such as missing sex dimorphism and smaller size at first−sex seem rather similar to those observed in *F. granulosa* (Kramarsky−Winter and Loya, 1998) than to those of *F. scutaria* (Loya and Sakai, 2008). Some of the largest and heaviest *F. concinna* individuals were females, but others were not reproductive (Figure 2.1, Figure 2.2). The apparent lack of reproduction in some large individuals could indicate that senescence occurs at a specific (weight, size or age) point in this species. This phenomenon has been demonstrated in the colonial coral *Stylophora pistillata* (Rinkevich and Loya, 1986), and also postulated for *F. granulosa* on the basis that no large individuals have been observed to spawn (Loya and Sakai, 2008). On the other hand, failure to reproduce could also be a consequence of stress effects or tissue damage during the reproductive season. In the case of *F. granulosa,* reproduction (albeit with reduced output) was maintained following tissue damage, but this could be a consequence of injury being sustained after the individual was committed to reproduction − in other words, once an individual is committed to gametogenesis, it may be unable to reallocate energy resources towards repair (Kramarsky−Winter and Loya, 2000), but that tissue repair might be enhanced after gamete release. As the largest specimen of *F. concinna* in this study reproduced as female, it is more likely that the lack of reproduction of some larger individuals was rather due to recovery after severe tissue damage than senescence.

This study is only the second to report bidirectional sex change in a fungiid. As sex change has been observed for solitary mushroom corals at a wide range of locations (Great Barrier Reef, Japan and the Red Sea), this phenomenon is likely to be common across the Fungiidae rather than a local adaptation (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). Based on similarities with *C. echinata* in reproductive traits, such as sex ratios and the relationship between sex and size, Loya and Sakai (2008) postulated the potential for bidirectional sex change also in *F. scutaria*. Fungiids were previously thought to be predominantly protandrous sequential hermaphrodites (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). However, the large number of female to male sex changes recorded in 2014, and the fact that some individuals in the smallest and lightest size classes were female, implies that protandry is not always the case in *F. concinna.* Although, as in other fungiid species, spawning time in *F. concinna* was tightly linked to season and lunar cycle, this species differed from *F. scutaria* and *C. echinata* in a number of other reproductive characteristics e.g. no clear relationship between size and sex and sex ratio (Loya and Sakai, 2008). It is difficult to draw conclusions on general reproductive traits for bidirectional sex change, as key characteristics are not yet identified. As size at first reproduction was similar between *F. granulosa* (Kramarsky−Winter and Loya, 1998) and *F. concinna* (this study), 55 mm and 50 – 75 mm (Figure 2.2), respectively, it is tempting to speculate that *F. granulosa* is also a sequential hermaphrodite. At 1:1.4, the overall ratio of females to males observed in *F. concinna* was similar to other fungiid corals (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). A recent study on the effect of aggregation on *F. concinna* showed that mucus production and movement were less frequent in conspecific than in heterospecific aggregations (Elahi, 2008). However, reductions in growth were observed irrespective of the aggregation composition, i.e. in terms of reductions in calcification rates, no differences were observed between *F. concinna* individuals surrounded by conspecifics or by *F. paumotensis*. It is possible that Fungiidae cannot only recognise 'who' is there but also what the sex ratio might be in a conspecific aggregation, and respond to changes in the sex ratio by either changing their own sex or remaining the same sex as in the previous reproductive season. The large number of female to male sex changes that occurred in the 2013 – 2014 period, combined with relying exclusively on corals that had been previously sampled suggests that the highly biased sex ratio observed in 2014 may have been a sampling artefact (Table 2.1).

When describing for the first time sequential hermaphroditism and bidirectional sex change in the hermatypic corals *Ctenactis echinata* and *Fungia repanda*, Loya and Sakai (2008) highlighted the similarities between plants and corals with respect to sex allocation. Sexual lability as observed in Fungiidae is a trait that in plants is associated with patchy environments varying in quality. Many dioecious plant species can alter their sex in response to changes in the ambient environment andfor changes in size or age (Freeman *et al.*, 1980). This lability of sexual expression could enable maximisation of the genetic contribution of an individual to the next generation (lwasa, 1991). As hermatypic corals, Fungiidae also acquire their daily energy requirements from their endosymbiotic zooxanthellae (Falkowski *et al.*, 1984). For the colonial coral, *Stylophora pistillata*, translocation of photosynthates derived from the endosymbiont provides the majority of energy resources required for gametogenesis (Rinkevich, 1989). Thus, as in plants, in at least some corals, photosynthesis is the ultimate source of energy for gametogenesis. These similarities in resource production led Loya and Sakai (2008) to suggest that fungiids could potentially enhance survival and reproductive success in similar ways in response to energetic andfor environmental constraints. lt has been suggested that, in the case of *C. echinata,* bidirectional sex change at intermediate size might be a mechanism allowing recovery from the relatively costly process of reproducing as a female (Loya and Sakai, 2008). The authors suggested that individuals of intermediate size may not have sufficient energy resources to reproduce as females every year. Hence it might be beneficial for the individuals to alternate sexes between years until they attain a critical size above which they are able to acquire sufficient energy to remain female (Loya and Sakai, 2008). The need for a recovery period after a major spawning event might also explain why some of the larger *F. concinna* individuals did not spawn in this study.

2.5.2 Gametogenesis

Following the observation of bidirectional sex change in *F. concinnca*, the question arose as to when the process of sex change was initiated. Random selection of two female and two male *F. concinna* individuals based on their sex during the previous spawning season, permitted the detection of bidirectional sex changes, one male to female and one female to male. In the former case, oogenesis had been initiated in February 2014 (i.e. three months after spawning), indicating that the signal for sex change must be given shortly after the release of spermatozoa. In March of 2014 (and February of 2015), oogenesis was at a similar developmental stage in the female that remained as female and the sex changer, indicating that oogenesis was initiated at the same time in both cases. With an onset of oogenesis in FebruaryƒMarch and spawning in late Novemberƒearly December, the oogenic cycle (9 – 10 months) in *F. concinna* is slightly longer than that (6 – 7 months) reported for the congeneric Red Sea species *F. scutaria* and *F. granulosa* (Kramarsky–Winter and Loya, 1998). Investigation of the nature of the signal for sex change was beyond the scope of this study due to time limitations. A recent study of gametogenesis in the gonochoric coral *Euphyllia ancora* (Shikina *et al.*, 2012) was able to correlate the onset of gametogenesis with expression of the germline marker *vasa*. The *vasa* mRNA was detected in oogonia, spermatogonia and early stage gametes and but not during later stages of gamete development (Shikina *et al.*, 2012). Expression of *vasa* and other germ line genes could also play an important role in *F. concinna* during sex change.

For the hard corals studied here, spawning after the full moon in the late Australian springƒearly summer at mid shelf reefs of the GBR had been described (Babcock *et al.*, 1986; Harrison and Wallace, 1990) and in the present study spawning was reliably observed in 2011 – 2014. Spawning of the alcyonacean coral *Lobophytum compactum* has also been observed at that time (Michalek–Wagner and Willis, 2001a, b). In the present study, however, *L. pauciflorum* colonies spawned predominantly one lunar month earlier, (i.e. after the October full moon) than was the case for the hard corals in both 2012 and 2013. Due to the full moon occurring early in October 2014, the average water temperature, 25.6 ± 0.27 ˚C, may not have been favourable for spawning in that month that year (2014), all of the *L. pauciflorum* colonies spawned after the November full moon, by which time the water temperature had reached 27 ± 0.52 ˚C. As the majority of the colonies sampled did not bear oocytes in late November, no observations were made after the December full moon in that year. Earlier reproduction of *L. pauciflorum* could be an advantage if suitable settlement substrate is limiting providing that environmental conditions at that time are permissive (Fan *et al.*, 2005).

On the other hand *L. pauciflorum* might be able to delay final maturation of oocytes if conditions are not suitable, e.g. lower water temperatures due to prolonged winter. The key signal for final maturation of coral oocytes is unknown. In the jellyfish *Spirocodon saltatrix*, final maturation of oocytes can be induced by a dark treatment and is completed within 2 h (Ikegami *et al.*, 1978). A similar light signal for final oocyte maturation could also be present in anthozoans. The exact timing of sunset a few days after full moon, for example, could be a trigger inducing gamete maturation. In the present case, both hard coral species studied contained gametes in late November but not in December. It is therefore likely that those corals spawned a second time after the December full moon. The potential for a split spawning further strengthens the idea of an induced final maturation of oocytes.

Average oocyte size differed greatly between the three species studied. Kahng and colleagues (2011) reported the normal average oocyte size for 119 shallow−water octocorals to be 686 ± 253 µm, whereas in *L. pauciflorum* the mean oocyte size was significantly smaller (190.00 ± 24.06 µm). However, the size of *L. pauciflorum* oocytes is in line with the average size of mature oocytes in the Japanese precious corals (280 − 360 µm; Nonaka *et al.*, 2015). On the other hand, oocyte counts were lower in October 2014 than in the previous month, a likely explanation being that larger oocytes might have been lost during the preparation procedure due to weak attachment to the mesenterial tissue. The loss of larger (mature) oocytes could therefore bias average oocyte size estimates in the present case. Immature oocytes were on average 203.2 ± 30.42 µm in September 2014, supporting the hypothesis of loss of mature oocytes from October 2014 samples during the fixation procedure. The previously reported average oocyte size sampled from the water column for *Acropora* spp. varied between 600 and 730 µm (Wallace, 1985; Babcock *et al.*, 1986). Kojis (1986) reported mean oocyte diameters for *Acropora cuneate* and *A. palifera* at Heron Island reef (South GBR), 255 ± 26.2 µm and 249 ± 38.8 µm, respectively, shortly prior to spawning. In the present study, *A. millepora* around Orpheus Island had mature oocytes measuring 257.60 ± 58.33 µm, with the largest oocyte being about 430 µm in November 2014. For both *A. millepora* and *L. pauciflorum,* the differences in oocyte diameter observed in this study to estimates based on samples from the water column could be a consequence of oocytes being compressed within tissue prior to release. For the solitary coral *F. concinna*, oocytes varied in size between 36.08 and 207.7 µm, with an average of 106.30 ± 28.88 µm in November 2014. These small oocyte size estimates compared to the two other species investigated are in line with published data for other fungiids, *F. granulosa* (130 ± 18.9 µm) and *F. scutaria* (126 ± 63.2 µm) (Kramarsky−Winter and Loya, 1998) and *Diaseris distorta* from the Galápagos Islands (104 µm Colley *et al.*, 2000).

Differences in orientation of the oocytes within the tissue or nutrient availability may also have contributed to discrepancies between the literature and estimated sizes of oocytes reported here.

Gamete development occurred primarily in the radial polyps of *A. millepora* rather than in the apical polyps, and predominantly in the siphonozooid polyps (with few exceptions, Figure 2.7) in the case of *L. pauciflorum*. Whereas gametogenesis was reported in the radial polyps with a zone of sterile radial polyps at the tips of most branches for *Acropora* spp. (Wallace, 1985), Simpson (2009) suggested that gametogenesis occurs exclusively in autozooid polyps in shallow−water octocorals. One male *L. pauciflorum* in this study was observed to produce spermatogonia in an autozooid polyp (Figure 2.7). Observations for *Lobophytum* here and studies in precious corals of Japan (Nonaka *et al.*, 2015) therefore appear to contradict Simpson's hypothesis but suggest that the sites of gametogenesis may vary across the Octocorallia. The observation of gametogenesis predominantly in siphonozooid polyps of *Lobophytum* appears to also represent a novel (additional) function for this polyp type.

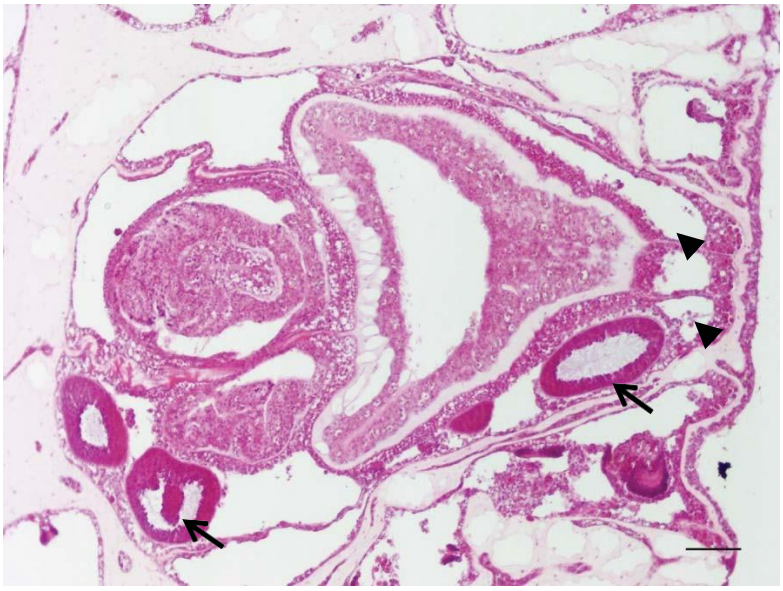


Figure 2.7 Example for an autozooid polyp with sperm sacs in *Lobophytum pauciflorum*, observed in September 2014, black arrows indicate sperm sacs and arrowheads point towards tentacles of the autozooid polyp. Scale bar represents 50 µm.

The sex ratio among the soft corals that were sampled on a monthly basis was heavily female biased. However for a different experiment, 17 colonies of *L. pauciflorum* were sampled from different locations around Orpheus Island in September 2014. Of these, nine colonies reproduced as male and 8 colonies as female during the spawning event in November 2014. Considering these additional colonies, it becomes clear that the biased sex ratio in this

study was an introduced sampling artefact and does not represent the actual population sex ratio, which is probably around 1:1 – as in the case of a Taiwanese *L. pauciflorum* population (Fan *et al.*, 2005).

The consensus is that oogenesis takes around 12 – 14 months in octocorals, whereas spermatogenesis is an annual event (Yamazato *et al.*, 1981; Alino and Coll, 1989; Benayahu, 1997a). Data presented here imply that, in *L. pauciflorum* on the GBR, oogenic cycles do not overlap. Even though the observation of large oocytes in January could be interpreted as evidence for overlapping gametogenesis cycles, larger oocytes were not observed later, implying that the larger oocytes observed in January were remainders from the spawning season that were ultimately reabsorbed. Reabsorption of unspawned material has previously been observed in the temperate gonochoric coral *Astrangia danae* (Szmant–Froelich *et al.*, 1980). In addition, the apparent absence of small (very early) oocytes (> 50 µm) immediately prior to spawning events supports the hypothesis that oogenesis is an annual event in *L. pauciflorum* on the GBR, and contrasts with reports of overlapping oogenic cycles in a Taiwanese population (Fan *et al.*, 2005). The duration of spermatogenesis was much shorter than was oogenesis in *L. pauciflorum*, which is in line with observations for other soft corals (Yamazato *et al.*, 1981; Alino and Coll, 1989; Nonaka *et al.*, 2015). Sperm development is possibly faster on the GBR than in the Taiwanese population, where it was described to take about 12 months (Fan *et al.*, 2005). However, the duration of spermatogenesis could not be established conclusively due to under–representation – the *L. pauciflorum* colonies studied here were predominantly female – and the fact that spermatogonia were only observed at a relatively mature stage of development. Spermatogonia were first observed in *L. pauciflorum* in late August, by which time the spermatozoa were arranged conspicuously at the periphery of the spermatocytes, with tails pointing towards the central lacunae (Figure 2.4 C). Thus the first observation of spermatogonia corresponds to descriptions as stage IV in spermatogenesis in Baird and co–authors (2011).

2.6 CONCLUSIONS

This is the second study to demonstrate bidirectional sex change in a fungiid species. Despite lacking clear sexual size dimorphism, in terms of other reproductive traits, such as lunar periodicity and gonochoric broadcast spawning, *F. concinna* resembles *Ctenactis echinata*, which is a protandrous sequential hermaphrodite capable of bidirectional sex change. As in *A. millepora* and *L. pauciflorum,* the onset of oogenesis preceded spermatogenesis in *F. concinna*. Gametogenenic cycles of the hard corals studied were of similar duration, with oogenesis starting in FebruaryfMarch and spermatogenesis in September for a spawning event in late November or early December. Oogenesis in *F. concinna* was found to be longer than has been reported for congeneric species in Japan and the Red Sea. Spermatogenesis, however, was of similar duration (6 − 8 weeks) to other Scleractinia. Oogenesis in *L. pauciflorum* was found to be an annual event, whereas overlapping oogenic cycles have been reported for other octocorals. On the GBR, spermatogenesis may also be of shorter duration in *Lobophytum pauciflorum* (2 − 3 months) than has previously been reported for other *L. pauciflorum* populations (about 12 months), highlighting the possibility of regional variability of gametogenesis. Gametes developed predominantly in siphonozooid polyps in *L. pauciflorum,* whereas it was previously thought to occur in the autozooid polyps of shallow−water soft corals. Furthermore, in two of the three years of this study, *L. pauciflorum* spawned a lunar month earlier than the major multi−species spawning events in which *A. millepora* and *F. concinna* participated.

The discovery of bidirectional sex change in a second fungiid coral (*F. concinna)* suggests that this life history trait − sequential hermaphroditism − might be more widespread than has been assumed. Clearly a great deal more work will be required to reveal the molecular mechanisms underlying sex determination and sex change in *F. concinna* and other fungiids. The apparent variability in the timing of spawning and duration of gametogenesis in *L. pauciflorum* suggests that it could be informative to conduct longer term observations across different locations along the GBR and elsewhere in order to better understand the links between physical conditions and reproduction in *Lobophytum*. By focusing on ecological aspects of reproduction in selected hard and soft corals species, this study highlights similarities and differences in the processes of gametogenesis and the timing of spawning, providing a basis for molecular analysis of sex determining mechanisms in hard and soft corals.

Chapter 3 Unravelling the morphological and molecular properties of soft coral early development in *Lobophytum pauciflorum*

This chapter represents the current state of the analysis. As spawning and fertilisation were only successful in the last two years of my candidature, I was unable to perform all the in depth analyses necessary for publishable format. Further results generated by *in situ* hybridisation will be included for publication. Once data collection will be finalised the chapter manuscript will be updated and submitted for publication. However, this chapter is inserted with an abstract for an identical format as the other data chapter.

Wessels W, Ball EE, Hayward DH, Miller DJ (to be submitted to Developmental Biology) Unravelling the morphological and molecular properties of soft coral early development in *Lobophytum pauciflorum*

All sampling and sample processing was done by W Wessels and E Ball. W Wessels was in charge of sample processing and analysis of the NGS part. E Ball prepared the fixed larval material for imaging and processed images. W Wessels wrote the chapter after intellectual contributions by all co−authors.

3.1 ABSTRACT

The early development of the hard coral *Acropora millepora* has been described at both the morphological and molecular levels, and comparative studies are under way for other Scleractinia. Complementary data on octocoral embryogenesis are essential for a broader understanding of the diversity and evolution of developmental strategies across the Anthozoa. The work presented here is pioneering in that it is the first combined study of the morphological and molecular bases of embryogenesis in a soft coral using *Lobophytum pauciflorum*. The morphology of early development in *Lobophytum* is very different to what has been reported for scleractinians or the sea anemone, *Nematostella vectensis*. Stages between the fertilised egg and the 16 – 32 cell morula consist of an irregular cell mass without obvious blastomeres. After blastomeres appear, successive rounds of cell division result first in smaller blastomeres, and with decreasing cell size the gastrula returns to an irregular mass of cells with a relatively smooth surface. Molecular data are crucial for a full understanding of this process in *Lobophytum* and its comparison to other Anthozoa. Thereto, transcriptome sequencing was carried out on unfertilised eggs, a range of morphologically distinct developmental stages and adult individuals. Using a *de novo* transcriptome assembly as reference, temporal patterns of differential expression were established relative to the adult stage. Analyses of these data imply that zygotic gene expression is initiated between 16 and 27 hpf, expression profiles at 16 hpf being more similar to unfertilised eggs and at 27 hpf more similar to the planula stage. On this basis, it is likely that gastrulation occurs during the 16 – 27 hpf interval. A preliminary analysis of genes that are differentially expressed during settlement and metamorphosis indicated that competent planulae already possess the basic molecular tool kit to induce calcification. However, more genes likely to be involved in calcification, including cytosolic carbonic anhydrases, were upregulated in settled polyps and adults.

3.2 INTRODUCTION

Early development within the Anthozoa has been described predominantly for the subclass of Hexacorallia, which includes both the hard coral *Acropora millepora* and the starlet sea anemone *Nematostella vectensis*. Descriptive embryology data have been complemented by molecular studies (e.g. Anctil *et al.*, 2007; Fritzenwanker *et al.*, 2007; Grasso *et al.*, 2008; Shinzato *et al.*, 2008). Despite increased interest in octocorals due to their chemical properties, little is known about the early development of Octocorallia compared to Hexacorallia. There is a small base for morphological descriptions of early processes (Wilson, 1883; Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998), but researchers have rather focused on later developmental processes such as infection with symbiotic algae, settlement and survival (Benayahu and Loya, 1987; Benayahu *et al.*, 1988; Benayahu *et al.*, 1989; Benayahu, 1991; Benayahu *et al.*, 1992; Lasker and Kim, 1996; Zaslow and Benayahu, 1996; Kruger *et al.*, 1998; Harii and Kayanne, 2003) than early developmental stages.

It is clear from the early literature that soft corals display a remarkable variety of early cleavage patterns (Wilson, 1883; Matthews, 1917). In many species, the first cytosolic division does not result in a 2−cell embryo but typically occurs after several rounds of nuclear division resulting in the appearance of 8 − 32 cell stage (Wilson, 1883; Matthews, 1917; Dahan and Benayahu, 1998). Successive rounds of cell division first lead to development of a spherical embryo that later becomes a pear shaped planula (Wilson, 1883; Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). In contrast to members of the Hexacorallia, octocorals lack morphologically characteristic stages between the blastula and the planula hence there are no clear indications as to when gastrulation occurs. Octocoral embryos are thought to gastrulate by delamination, *i.e.* one cell layer splits or migrates of to form two cell sheets (Wilson, 1883; Matthews, 1917).

Gastrulation in octocorals leads to the development of an early planula that is pear−shaped and sparsely flagellated (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Mature planulae are slender with a broad oral end and a tapered aboral end, have a densely flagellated surface and swim actively in the water column (Matthews, 1917; Benayahu and Loya, 1986; Uehara *et al.*, 1987; Benayahu *et al.*, 1988; Dahan and Benayahu, 1998). In the azooxanthellate octocoral *Dendronephthya hemprichi* from the Red Sea (Dahan and Benayahu, 1998) mature planulae are immediately competent to settle. However, planulae of most species spend some time in the water column prior to settlement (Matthews, 1917; Uehara *et al.*, 1987; Benayahu, 1989) and remain competent to settle for an extended

period of time (Benayahu and Loya, 1986; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Even in *D. hemprichi* settlement is asynchronous and some planulae continued to swim in the water column before settling for up to 2 days after the first planulae settled (Dahan and Benayahu, 1998). Once they are competent, planulae attach to the substrate via the tapered aboral end (Uehara *et al.*, 1987), become progressively shorter and metamorphose (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Metamorphosis into a primary polyp with 8 tentacular buds occurs within the first day after settlement (Matthews, 1917; Uehara *et al.*, 1987; Dahan and Benayahu, 1998). Tentacular buds of primary polyps continue to grow and develop into moveable tentacles, which eventually also become pinnate (Matthews, 1917; Uehara *et al.*, 1987). Sclerites were observed in the polyp body as early as the second day after metamorphosis in *Alcyonium digitatum* (Matthews, 1917), whereas sclerite formation took 3 – 4 days in *D. hemprichi* (Dahan and Benayahu, 1998) and two weeks in *Lobophytum crassum* (Uehara *et al.*, 1987).

In octocorals, sclerites composed of calcite crystals are localised in the mesoglea and in some species also in the axis. Sclerites function in physical support and defence against predators (Van Alstyne *et al.*, 1992). Due to the lack of an exoskeleton, alcyonacean octocorals are generally not considered to be reef–building corals. However some *Sinularia* species do contribute to reef structures by consolidating sclerites at the colony base forming spiculite and amorphous calcium carbonate (Jeng *et al.*, 2011). In the case of gorgonians, calcification is described as initially intracellular, followed by an extracellular calcification phase (Goldberg and Benayahu, 1987; Grillo *et al.*, 1993). In the intracellular step, electron lucent vesicles are formed within the scleroblasts. These vesicles then calcify and grow before forming one or more intracellular masses of polymorphic crystals that are released to the mesoglea (Goldberg and Benayahu, 1987). In the extracellular space, the mesoglea, secondary scleroblasts attach to these sclerite primordia and produce layers of calcite crystals with irregular proturbances (Goldberg and Benayahu, 1987; Grillo *et al.*, 1993). As a result, several of those primordia are integrated into a single immature sclerite, which continues to grow by calcite deposition of secondary scleroblasts (Goldberg and Benayahu, 1987). This process was confirmed for soft corals in the Alcyoniidae family by Jeng and colleagues (2011). At the molecular level, extracellular proteins capable of inducing calcite formation in vitro and organic matrix proteins including carbonic anhydrases, an actin isoform and a previously unidentified protein 'scleritin', have been implicated in soft coral calcification (Lucas and Knapp, 1996; Rahman *et al.*, 2007; Rahman *et al.*, 2011; Debreuil2*e*0*t*1*a*2*l*.;„Rahman *et al.*, 2013).

In hexacorallians such as *N. vectensis* (Fritzenwanker *et al.*, 2004; Kraus and Technau, 2006) and *A. millepora* (Babcock and Heyward, 1986; Ball *et al.*, 2002b) the predominant mode of gastrulation is invagination. By contrast with octocorals, in both of these hexacorals gastrulation is a morphologically distinct process, in the case of *Acropora* from the concave–convex 'prawn chip' stage, whereas in *Nematostella* gastrulation occurs from a near–spherical blastula (Hayashibara *et al.*, 1997; Miller *et al.*, 2000; Fritzenwanker *et al.*, 2004; Kraus and Technau, 2006). Post–gastrulation development also leads to a planula stage in both *N. vectensis* and *A. millepora*, which metamorphoses into a primary polyp 5 – 10 days and 4 – 7 days post–fertilisation, respectively (Babcock and Heyward, 1986; Kraus and Technau, 2006). Close investigation of the gastrulation process in *N. vectensis* enable the identification of organising activity in the blastopore lip similar to processes in vertebrates (Kraus *et al.*, 2007). Expression of homologous genes known to play crucial roles in bilaterian gastrulation, in particular secreted factors, in this blastoporal region (Matus *et al.*, 2006; Rentzsch *et al.*, 2006) strongly suggests a common origin for the cnidarian and bilaterian blastopores (Kraus *et al.*, 2007). Comparing the localisation of expression of key organiser genes between *N. vectensis* and *A. millepora* revealed that expression patterns were generally similar (Hayward *et al.*, 2015). Despite the large similarities between *A. millepora* and *N. vectensis*, gene expression patterns of *brachyury*, identified as key player for the differentiation between ecto– and endoderm in *A. millepora*, need to be further explored in the starlet sea anemone as *brachyury* expression is co–localised with *forkhead* expression, complicating clear identification of a germ layer differentiator in *N. vectensis* (Fritzenwanker et al., 2004; Hayward et al., 2015). Despite varying time and morphology between complex and robust Scleractinia, orthologs showed similar expression patterns to those seen in *A. millepora* and *N. vectensis* (Okubo *et al.*, 2016). However all three coral species investigated by Okubo and colleagues (2016) displayed early *forkhead* expression, which was not reported in *N. vectensis*. The fact that homologous genes might be deployed differently between related Hexacorallia indicates that we are still far from understanding cnidarian development and the evolution of this process. In order to get a broader view on modes of gastrulation and the involved candidate genes in Anthozoa, comparative data from Octocorallia are crucial for future comparative studies.

The scleractinian exoskeleton consists of aragonite crystals. However, the process by which this exoskeleton is produced is still under debate (reviewed by Allemand *et al.*, 2004; Allemand *et al.*, 2011). The two contrasting hypotheses are 'biologically induced mineralisation' (Lowenstam, 1981) and 'biologically controlled mineralisation' (Lowenstam, 1981; Mann, 1983). In *Pocillopora damicornis*, metamorphosis is tightly linked to settlement and within a few hours of settlement the larval epidermis is replaced by a simple epithelium

consisting of only calicoblast cells (Vandermeulen, 1975). The first rudimentary elements are then formed within 6 h post settlement and fuse to form the basal disc within 48 – 72 h (Vandermeulen and Watabe, 1973). In contrast to Octocorallia, the consensus is that in Scleractinia calcification is induced extracellularly (Johnston, 1980; Allemand *et al.*, 2011). The observation of intracellular vesicles containing calcium and organic matrix within calicoblastic cells suggests that exocytosis is involved in the secretion of organic matrix in hard corals (reviewed by Allemand et al., 2011). The current hypothesis is that calcification is a two–step process in Scleractinia (Cuif and Dauphin, 2005, Cuif 2008). Allemand and colleagues (2011) propose that the calicoblastic cells first secrete a nanosized organic cortex – the 'nanocortex' – which becomes enriched with ionic calcium or amorphous calcium carbonate. Through a series of chemical reactions, the calcium ions then crystallise with carbonate ions to from aragonite (Allemand *et al.*, 2011). Whilst this is an elegant hypothesis, many pieces of the puzzle remain hypothetical. Galaxin was the first protein identified within the organic matrix of adult *Galaxea fascicularis* and is characterised by dicysteine repeats potentially enabling crosslinking of this protein and thus the formation of a macromolecular network (Fukuda *et al.*, 2003). Reyes–Bermudez and colleagues (2009) identified 3 galaxin related sequences containing dicysteine–rich repeats in *A. millepora*. These 3 galaxin related sequences showed different expression patterns throughout *Acropora* settlement: while *Amgalaxin* was highly expressed in post–settlement stages and adults, *Amgalaxin–like 1* and *2* were exclusively expressed during the onset of calcification in pre–settlement stages (Reyes–Bermudez *et al.*, 2009). The restricted expression of *Amgalaxin* to adults was consistent with the expression pattern observed in *G. fascicularis* where Galaxin expression was only identified in adults but not in planulae (Fukuda *et al.*, 2003). Another group of proteins implicated in calcification are carbonic anhydrases (CAs), some of which are specifically expressed in the calicoblastic epithelium in scleractinian corals (Hayes and Goreau, 1977). As CAs promote the hydration of carbon dioxide they are believed to be crucial for the increase of carbon dioxide hydration in the nanocortex (Allemand *et al.*, 2011). By sequencing the proteome of the *A. millepora* organic matrix, Ramos–Silva and colleagues (2013) identified 36 skeletal organic matrix proteins (SOMPs) including secreted proteins such as Galaxin and extracellular regions of transmembrane proteins. The diversity of proteins identified highlighted the complexity of the organic matrix; it is not composed simply of acidic proteins, but consists of a wide range of proteins with a large spectrum of potential functions. The authors further suggested that not all SOMPs are secreted but some are membrane–bound proteins (Ramos–Silva *et al.*, 2013). Despite recent progress in the identification of proteins in the organic matrix and the

calicoblastic epithelium, their roles in coral calcification remain to be clarified (Allemand *et al.*, 2011).

The work presented here is the first to combine large–scale gene expression analyses with morphological data on embryogenesis and early development in a soft coral. The integration of morphological and molecular analyses in *Lobophytum pauciflorum* not only provides preliminary insights into the molecular bases of soft coral development but also enables comparative developmental analyses between Hexacorallia and Octocorallia. Of particular interest are molecular events underlying gastrulation and metamorphosis, processes for which some molecular data are available from hexacorallians but not for any octocoral. As metamorphosis is coupled to the initiation of calcification, we hope to gain insights into how soft corals differ from hard corals in terms of the initiation of calcification. Contrasting calcification in hard and soft corals is of specific interest in the context of understanding the biology underlying precipitation of calcium carbonate as either aragonite (as in hard corals) or calcite (as in soft corals). Comparison of expression patterns using in–situ hybridisation should increase understanding of the molecular bases of the broad range of developmental strategies displayed by Anthozoa.

3.3 MATERIALS & METHODS

3.3.1 Animal collection, maintenance and sampling

Lobophytum pauciflorum colonies were collected from reefs adjacent to Orpheus Island (18˚34' S; 146˚29' E) from September to November 2014 two nights after the monthly full moon, in accordance with the GBRMPA permit G12ƒ35295.1. Colonies were maintained in a 1000 L flow through system at Orpheus Island Research Station (OIRS) after the collection. Prior to spawning, colonies were isolated to allow sex determination and targeted gamete collection. In addition to *in aquaria* collection, *in situ* oocyte collection was performed. Collection nets were placed over designated colonies two hours prior to spawning on night 4 and 5 after full moon (Appendix Figure 3.1), and retrieved 30 min after spawning. As oocyte numbers were too low to permit single crosses, one fertilisation mix was set up by combining *in situ* collected oocytes from 8 colonies with sperm collected from 4 colonies at OIRS in 1 µm filtered sea water. After 6 h, developing embryos were washed twice with filtered sea water and transferred to a 500 L rearing tank in a temperature controlled room with water temperature set at 27.5 ˚C. 24 h post fertilisation, air and very low flow through were introduced to the rearing tank to provide the larvae with fresh water and avoid increased death due to low oxygen availability and parasite growth.

The developmental process was closely monitored by regular sampling from the pool and assessment of developmental progress and larval health under the microscope. At regular intervals, samples were taken for RNAseq and histology. For RNAseq, triplicate samples each of 100 larvae taken from the above pool were collected in cryo tubes (Greiner, Australia), snap frozen in liquid nitrogen and stored at −80 ˚C. For settlement stages, 5 − 20 polyps were pooled, as the number of settlers at a similar developmental stage was limited. Induction of settlement was tested with water and ethanol extracts of both adult tissue and crustose coralline algae (CCA) at the base of adult colonies. Further attempts were made to induce settlement using different concentrations and incubation times of CsCl. The highest settlement rates were obtained when larvae were incubated for 5 h with 58 µM CsCl. After incubation, larvae were washed carefully and thoroughly to remove any residual CsCl and transferred to a new settlement dish. Larval survival was closely monitored after CsCl treatment and water exchanged daily. Settled larvae were sampled after varying length of settlement time, 1 day − 3 weeks. At the same time as RNAseq samples were taken, samples for morphological analysis of the developmental processes and *in situ* hybridisation were taken following methods described by Anctil and colleagues (2007).

3.3.2 RNA extraction using illustra RNAspin Mini kit (GE Healthcare Life Sciences)

For RNA extraction and sequencing, 8 developmental time points were selected: 16 hpf (blastula), 27 hpf (late gastrulaſearly sphere), 36 hpf (unmotile planula), 48 hpf (motile planula), 96 hpf (swimming planula), 183hpf, 48hps (upside down swimming), 1 wps (young polyp) and 3 wps (large polyp) based on their morphological properties (see 3.4.1 for more details). In addition to the developmental stages, unfertilised eggs and 2 adult males were also processed for RNA sequencing as 'start' and 'end' points for developmental comparison.

Prior to RNA extraction, 350 µL of RNAlater®–ICE (Invitrogen, Life Science Technologies, Australia) were added to the larvae and allowed to react for 24 h at −20 ˚C. Larval RNA was extracted using the illustra RNAspin Mini kit (GE Healthcare, Australia) according to supplier's protocol and eluted twice in 40 µL of nuclease−free water. To further concentrate the samples, RNA was precipitated using ethanol and sodium acetate (3 M, S7899, Sigma−Aldrich, Australia) and resuspended in 25 µL nuclease−free water. RNA concentration and purity were analysed using a NanoDrop ND−100. The purity and integrity of RNA samples was also verified on a denaturing gel using standard methods (Sambrook & Russell 2011). RNA samples were stored at −80 ˚C prior to shipping to the Australian National University for cDNA library preparation and sequencing. As previous studies on *Acropora millepora* have shown that biological replicates are unnecessary in experiments that involve a large number of different genotypes (Moya *et al.*, 2012), single libraries per sample were sequenced.

3.3.3 Transcriptome sequencing, assembly and annotation

Aliquots of 1 − 1.5 µg of total RNA prepared from developmental stages were sent to the Australian Cancer Research Foundation (ACRF) Biomolecular Resource Facility (BRF) at the Australian National University in Canberra for library preparation and sequencing. mRNA libraries were prepared from total RNA via purification of PolyA RNA. Sequencing was carried out by running the pooled libraries in 2 lanes on an Illumina HiSeq2000 platform, collecting 100 base pair (bp) paired end data. At the first attempt, the 96 hpf and 183 hpf stage libraries were seriously underrepresented; in a second run, all developmental stages were included and data from both runs (i.e. from 4 HiSeq lanes) were combined for the analysis. Processing of output sequences was performed following broadly the protocols developed by De Wit et al. (2012). Sequences were cleaned for Illumina adaptor sequences and individual barcodes and selected for a quality score of 30 and minimum length of 70 bp. Cleaned sequences were sorted into paired and single reads and assembled into a reference transcriptome using Trinity and the normalisation function (Haas *et al.*, 2013). Transcripts of 300 bp length or longer were

considered for further sorting into host and symbiont sequences using the psytrans algorithm written by Sylvain Forêt and Jue−Sheng Ong (https:ƒƒgithub.comƒsylvainforetƒ). Sequence data for *Symbiodinium* sp. clade C, which is the most abundant clade in *Lobophytum* spp. and in octocorals in general on the GBR (Goulet *et al.*, 2008a; Goulet *et al.*, 2008b), were provided by S. Forêt from a previous transcriptome assembly for *Fungia concinna*. Due to time constraints and the focus of this thesis chapter on the host development, the symbiont fraction of the transcriptome was not annotated. Coding regions in the host transcripts were predicted using TransDecoder and annotated with Trinotate. Both are built in tools within Trinity (Haas *et al.*, 2013). Contig sequences and predicted protein sequences were blasted (blastx and blastp, respectively) (Altschul *et al.*, 1990) against the SwissProt database (The UniProt Consortium, 2015). Protein domains were identified using HMMER and PFAM (Finn *et al.*, 2011; Punta *et al.*, 2012). In addition transmembrane domains and signal peptides were predicted with tmHMM and signal, respectively (Krogh *et al.*, 2001; Petersen *et al.*, 2011). Gene ontology (GO) annotations, functional pathways and orthologous groups were identified using the GO, KEGG and eggnog databases (Ashburner *et al.*, 2000; Kanehisa *et al.*, 2011; Powell *et al.*, 2011).

3.3.4 Differential expression analysis

The reads used for transcriptome assembly were mapped against the host transcriptome with bowtie2 (version 2.2.4, Langmead *et al.*, 2009). The reads from each lane were mapped individually and treated as technical replicates for the developmental stages. Reads for the 2 adult samples were used as true biological replicates. The reads mapping to multiple contigs were then used as a proxy to assess sequence similarity and expression levels of the contigs using Corset (Davidson and Oshlack, 2014). Contigs with less than 10 mapped reads were discarded from the analysis. The resulting cluster counts were used to identify differentially expressed genes using the edgeR package in R (Robinson *et al.*, 2010). Counts from each lane were combined for each developmental stage, except for the adult stage, as these samples were genuine replicates. Cluster counts were normalised using RLE. Clusters were filtered for 10 counts per million (cpm) in at least 3 libraries. As no biological replicates were available and each sample was drawn from a pool of genotypes, the biological coefficient of variation was first estimated at 0.19 and then conservatively set to 0.4 based on recommendations in the edgeR user guide (Chen *et al.*, 2008). For the purposes of analysis, differential expression throughout development was calculated based on the adult expression levels. Only those clusters with a false discovery rate (FDR) < 0.05 were considered for annotation and gene ontology enrichment (GO). GO enrichment was analysed with the GOseq package (Young *et al.*, 2010), which takes gene size and expression level into account.

3.4 RESULTS

3.4.1 Morphological description of early development in *Lobophytum pauciflorum*

After establishing the most effective gamete sampling technique for *L. pauciflorum*, fertilisation experiments were successfully performed in November 2014 and 2015. Determination of morphological characteristics at specific time points in the development of *L. pauciflorum* was challenging, as development was asynchronous and at any one time morphologically highly diverse (Figure 3.1). There were no visible indications of first cleavage, no polar bodies were extruded or and no clear signs of successful fertilisation were apparent. Prior to the appearance of obvious blastomeres (Figure 3.1 C), fertilised eggs underwent a series of shape changes, some assuming polygonal shapes, some with ridges appearing (Figure 3.1 A and B), before returning to a near spherical shape. Stages between fertilised eggs and 16 – 32–cell embryos consisted of irregular cell masses without any obvious blastomeres (Figure 3.1 A, B and D). In the absence of any more appropriate marker, the appearance of 16 – 32–cell embryos between 3 – 5 h post fertilisation (hpf) was used as the internal control for a successful fertilisation (Figure 3.1 C and D).

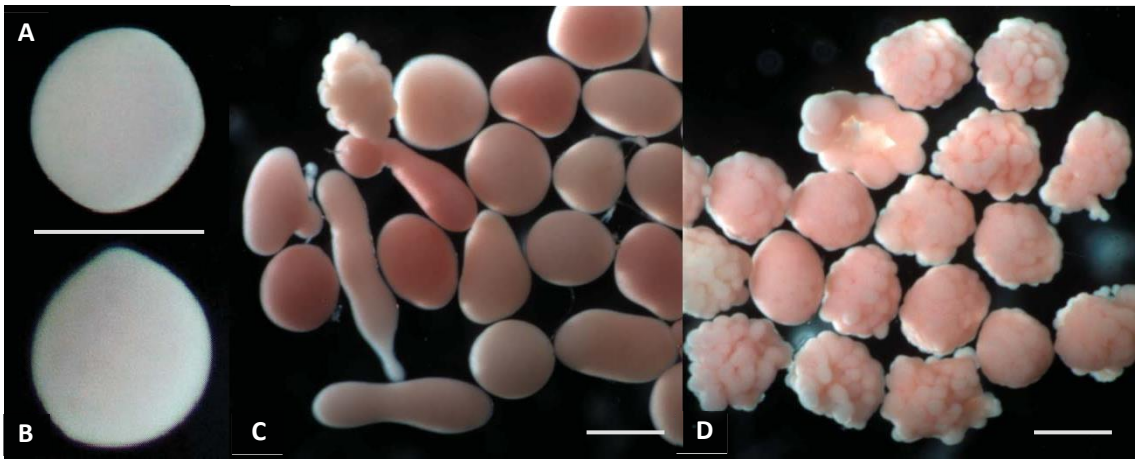


Figure 3.1 Developing *Lobophytum pauciflorum* embryos. A) At 3 h post fertilisation (hpf) oddly shaped embryos without any signs of cleavage activity dominate whereas B) at 5 hpf embryos with clearly distinguishable blastomeres are present. Scale bar represents 500 µm.

Successive rounds of cell division followed the 16 – 32 cell embryos, first creating smaller blastomeres and then, as the cells became smaller, returning to an irregular mass of cells with a relatively smooth surface (Figure 3.3, 8 – 13 hpf). By 36 h, pear shaped, non–motile, planulae had developed, and these began swimming within the following 12 h. The appearance of planulae and onset of swimming behaviour was more synchronised than was earlier development. *L. pauciflorum* larvae remained in the planulae stage for about 4 days (Figure 3.3), after which they began swimming downwards when taken out of the rearing tank

and transferred into a petri dish. However, in 2015 one planula was observed to be competent to settle at 96 hpf, and had metamorphosed within 24 h of settlement, *i.e.* by 120 hpf, a primary polyp had developed. Calm water conditions induced planulae to stop active swimming and move into a hovering position with the tapered aboral end facing the bottom of the dish. In this hovering position, planulae were sensitive to any movement of the petri dish, which resulted in the planulae returning to active swimming behaviour.

The majority of planulae remained swimming in the water column of the rearing tank or the petri dish for up to 7 days. At that stage all remaining planulae from the rearing tank were transferred to petri dishes to facilitate settlement. Incubation with 58 µM CsCl resulted in planulae rounding up into a spherical shape. However, after thorough washing following the incubation, planulae returned to the elongated shape and resumed swimming activity. After a few hours, planulae treated in this way slowed down and returned into the vertical hovering position. Hovering larvae eventually made contact with and attached to the dish, forming a mound–like shape. Metamorphosis occurred within the following 12 – 24 h after settlement, however only a small number of planulae metamorphosed while the remaining planulae remained in the water column and eventually died (Figure 3.3). After successful metamorphosis, the primary polyps had 8 small tentacular buds that fully developed into mobile tentacles that became pinnate within 1 week post settlement (wps) (Figure 3.2, arrowheads) and sclerites were observed within the polyp tissue at approximately the same time (Figure 3.2, arrows). By 3 wps, the 8 elongated tentacles had become pinnate and were actively moving, possibly in order to catch food particles.

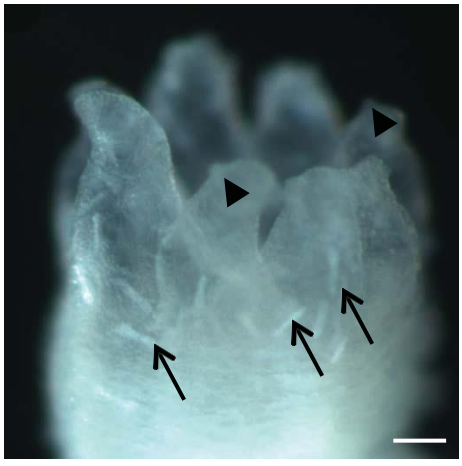


Figure 3.2 *Lobophytum pauciflorum* polyp 1 week post settlement (wps). Black arrows point towards sclerites and arrowheads indicate pinnate development of the tentacle tips, scale bar represents ~500 µm (Photo: Eldon Ball).

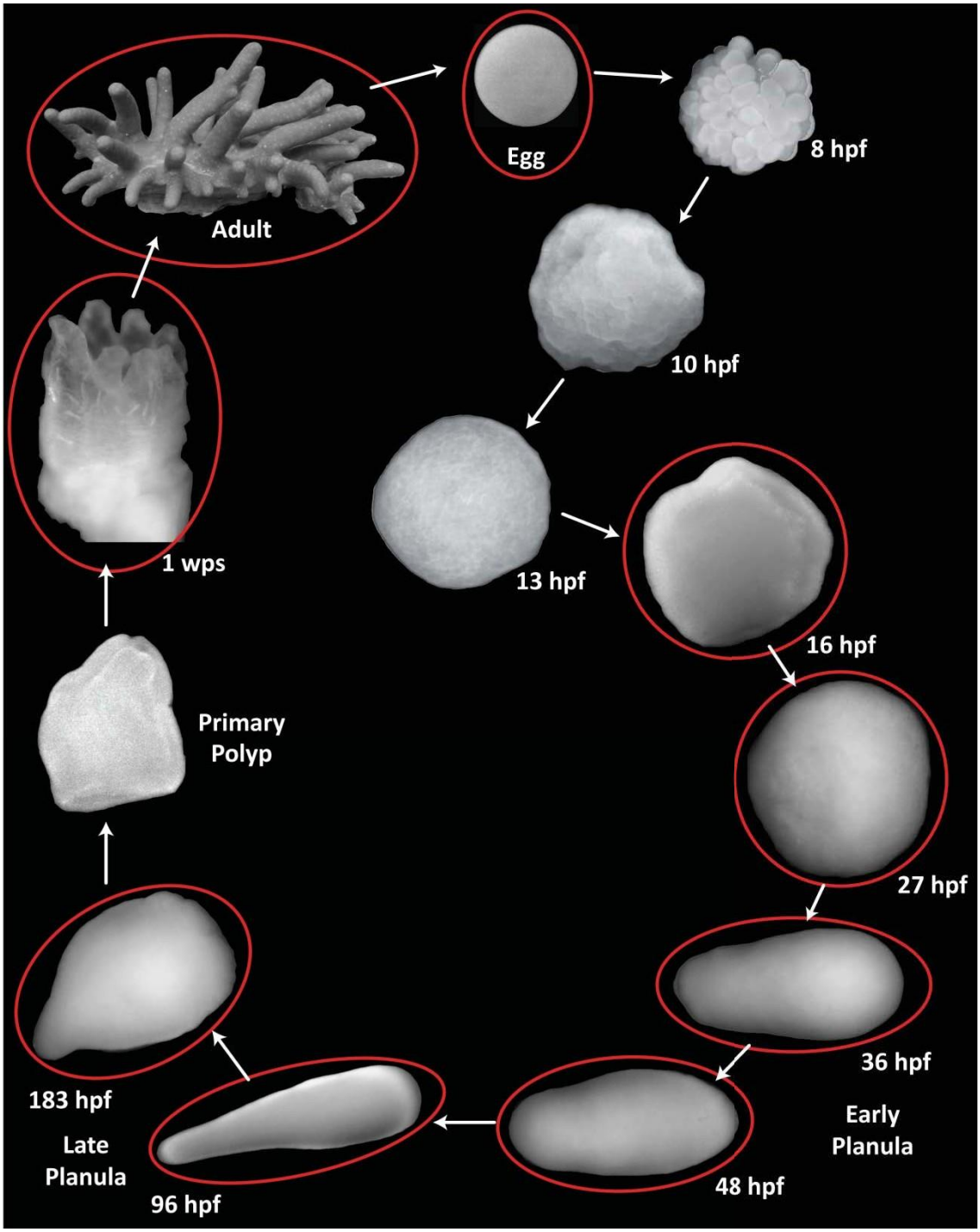


Figure 3.3 Light micrographs of developmental stages in *Lobophytum pauciflorum*. Stages with red circles were used for RNAseq, in addition a second polyp stage 3 wps was used for sequencing. Please note developmental stages are not to scale (Photos of 16 hpf – 183hpf, 1wps: Eldon Ball).

3.4.2 Transcriptome sequencing and assembly

Sequencing of 11 samples, corresponding to 10 developmental stages (reads of adults were combined), resulted in a similar coverage for each stage (Table 3.3), although the two late planula stages (96 and 183 hpf) had somewhat lower coverage for reasons outlined in methods section 3.3.3. The libraries prepared from the two adult samples were sequenced together on one lane. The majority of reads passed stringent quality checking, with only about 20 % of the reads remaining as singletons (Table 3.3). After read normalisation, the *L. pauciflorum* database contained 98376876 single sequences, which could be assembled into 632920 contigs with an average length of 803.04 bp, N_{50} of 1366 bp and a GC content of 41.04 % (Table 3.1). Of these contigs, 435925 (68 %) were 300 bp or longer. Average contig length and N_{50} were 878.89 bp and 1192 bp, respectively. The host fraction of the transcriptome comprised 359799 contigs (Table 3.1) with an average length of 1044.74 bp and a N_{50} 1587 bp. The symbiont component contained 76126 contigs with an average length of 1110.51 bp and a N_{50} of 1450 bp. The GC contents of the host and symbiont fractions were 38.14 % and 54.69 %, respectively. After extraction of only the longest isoform per contig, the transcriptome comprised 232830 contigs (Table 3.1). On average contigs were 884 bp long, 50 % of the contigs were longer than 1233 bp, and the GC content was 37.95 %.

Table 3.1 Summary of assembly statistics of different transcriptome versions/fractions

	All sequences	Sequences > 300 bp	Host fraction > 300 bp	Symb. fraction > 300 bp	Longest isoform per 'gene'
Number contigs	632920	435925	359799	76126	232830
Average contig length (bp)	803.04	878.89	1044.74	1110.51	884
N_{50} (bp)	1366	1192	1587	1450	1233
GC content	41.04 %	41.04 %	38.14 %	54.69 %	37.95 %

The annotation results for the longest isoforms of the host transcriptome fraction were used to extend the results for all isoforms (Table 3.2). 20 % of the longest isoform were found to have a match in the SwissProt database andfor conserved domain in the Pfam, TmHMM or SingalP database. About 37 % of the blast hits also had a GO description, although only about 4 % of the contigs were annotated with the SwissProt database. Using the annotations of the longest isoforms to annotate the remaining isoforms, lead to an annotation of about 30 % of the entire host transcriptome fraction.

Remapping of the reads that were used for the transcriptome assembly against the assembled host transcriptome resulted in coverage that varied between 62 % and 82 %. Mapping coverage was slightly lower in adult samples than in the developmental stages.

Table 3.2 Overview of sequence database for *Lobophytum pauciflorum*

	Longest isoform per 'gene'	**All sequences > 300 bp**
Number of all contigs	232830	359799
Contigs with BLAST hits	10054	28566
Combination with GO description	3722	11669
Contigs with Pfam and SignalP, TmHMM hit	10316, 4332, 9981	27494, 9504, 21370
Contigs without hits	185815	240839
% annotated	20.19	32.88

Table 3.3 Sequencing and mapping statistics of RNAseq experiment in *Lobophytum pauciflorum*. hpf = hours post fertilisation, wps = weeks post settlement.

	Unfertilised eggs	16 hpf	27 hpf	36 hpf	48 hpf	96 hpf	183 hpf	1 wps	3 wps	Adult
Raw Reads	132976182	160680162	236225766	227845622	222426302	82110552	61983348	177036800	158699922	377942394
Cleaned Reads	82.40%	81.88%	82.30%	82.30%	83.01%	82.15%	81.99%	82.29%	82.21%	80.35%
Single Reads	16.77%	17.66%	17.26%	17.29%	17.08%	17.66%	17.83%	17.18%	17.23%	15.36%
Paired Reads	41.62%	41.17%	41.37%	41.35%	41.46%	41.17%	41.08%	41.41%	41.39%	36.26%
Reads mapped	77.30%	81.00%	81.68%	81.60%	80.07%	81.00%	81.07%	77.59%	78.08%	62.03%

3.4.3 Temporal gene expression patterns compared to adults

Of the 360,000 contigs that comprised the transcriptome, 26561 were discarded from the expression and clustering analysis, as less than 10 reads mapped to them. The remaining 93 % of mapped reads were grouped into 186505 expression clusters. 21275 of these clusters met the selection criteria of 10 cpm in at least 3 libraries. Of these clusters, 1573 (comprising 4789 contigs) were differentially expressed throughout development relative to the adult stages at FDR < 0.05. The temporal patterns of differential expression could be gathered into approximately 7 expression groups, G I – VII (Figure 3.4 A). Generally, gene expression patterns appear to be most adult–like in the two polyp stages, as the majority of contigs were only slightly up or down regulated. Only a selected set of contigs was strongly upregulated, mainly contigs in groups G I and IV (Figure 3.4 A).

Expression groups I – IV accounted for 34 % of the differentially expressed contigs and typically the contigs in these groups were upregulated during development relative to the adult expression levels. In groups I and III, expression increased through development and contigs were a) highly upregulated from early planula stages to polyp stages (G I), or b) expression peaked during planula stages and then decreased in polyp stages (G III). For example, over–expression of contigs involved in the regulation of cell growth and RNA–DNA hybrid ribonuclease activity started after 16 h of development (G III, Figure 3.4, Table 3.4). Expression increased until 183 hpf and then began to decrease in the polyp stages, though levels still remained higher compared to adults. Expression in groups II and IV was high in the unfertilised eggs and either a) decreased over time through development (G II), or b) was high throughout development but decreased at the 3 weeks post settlement (wps) stage (G IV). Among the sequences in expression group II were 5 contigs closely related to homologs of the dispatched gene (disp) (Q6R5J2.1, 2 contigs; Q9P2K9.2, 3 contigs) deposited in the SwissProt database. disp–like sequences in *L. pauciflorum* were about 4 times more abundant in unfertilised eggs than in adults whereas these contigs were less abundant during all other developmental stages studied here.

By contrast with groups I – IV, contigs in expression groups V – VII were typically more highly expressed in adults than during development. Group V was the largest of the seven groups recognised and comprised over 40 % of the differentially expressed contigs. In this case, contig expression was typically lowest in unfertilised eggs, increased with ongoing development to become maximal in the adult stage. Levels of contigs in group VI were at their lowest levels in both unfertilised eggs and at 16 hpf. Expression of the majority of the contigs in group VI increased after 16 hpf and a second increase occurred after the late planula stage.

Contigs of group VII were at low levels at all stages to 183 hpf. After settlement, 1 wps, these contigs were upregulated compared to the previous developmental stages but expression levels were still lower compared to adults. One dominant GO function in G VI of particular interest was Wnt signalling, prompting an overview of Wnt ligands across the developmental stages. *Lobophytum* sequences closely related to Wnt1 and 3a (P21551.1, 4 contigs; Q2LMP1.1, 1 contig, respectively) were almost 4 times more abundant in unfertilised eggs than in adults whereas expression levels were lower in the other developmental stages (G V, Figure 3.4, Table 3.4). Wnt5a related sequences (O13267.1) were more abundant among adult sequences than in all developmental stages (G VI, Figure 3.4, Table 3.4).

Based on comparison with the SwissProt database, 70 % of the *Lobophytum* transcripts had no significant matches and hence carry no annotation (Table 3.2). Likewise, few of the differentially expressed *Lobophytum* contigs had cnidarian–specific matches (Figure 3.4 B). Contigs with only cnidarian hits were scarce and generally only a few were present in each expression group. Cnidarian hits made up about between 1 – 3 % of all hits in all groups, except for G IV in which none were observed. Generally unannotated contigs were the most abundant, and sequences with annotations to other non–Cnidaria made up only a third or half of the all contigs in the expression groups. However, in G II, G V and G VI the ratio between *Lobophytum* specific and common was about 1:1 (Figure 3.4 B).

Table 3.4 Dominant gene ontology (GO) functions of the expression groups. n=4789

	Number of contigs	Number of overrepresented GOs	Dominant GO functions
G I	234	6	Ion channel activity, ion transport, exonuclease activity
G II	952	51	Methyltransferase activity, hedgehog receptor activity, neuropeptide signalling pathway,
G III	397	8	Regulation of cell growth, RNA–DNA hybrid ribonuclease activity, polysaccharide binding
G IV	62	15	Transmembrane transport, ion channel activity, ion transport, sequence–specific DNA binding
G V	2093	65	Calcium ion binding, carbohydrate metabolic process, homophilic cell adhesion
G VI	1016	51	Regulation of apoptosis process, Wnt signalling pathway
G VII	35	3	Serine–type endopeptidase activity, proteolysis, membrane

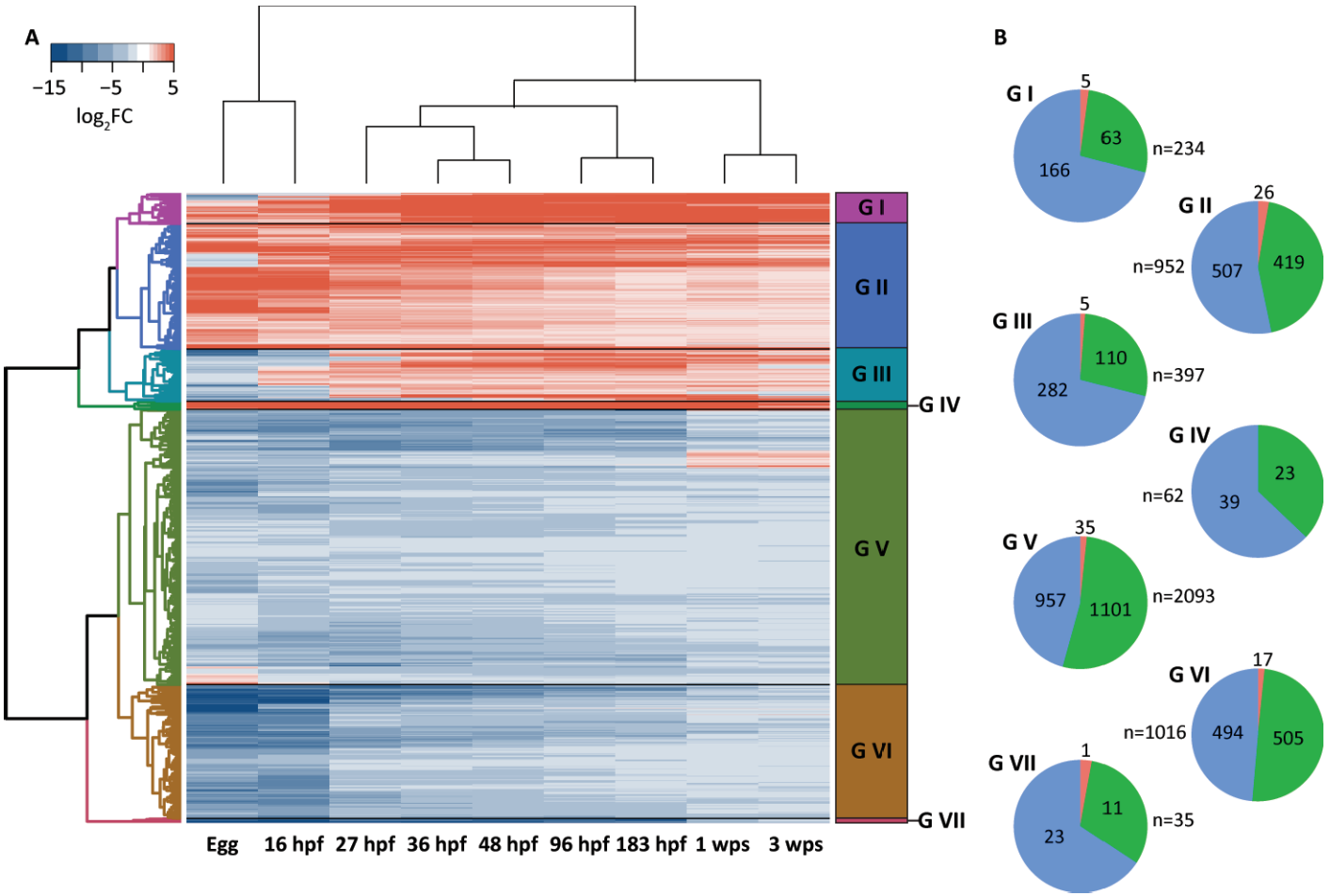


Figure 3.4 (A) Heat map of 4789 differentially expressed contigs relative to adults with a FDR of Š 0.05 and a log_2FC Š -1 or Š 1. The more intense the blue the more downregulated the expression, the more intense the red, the higher the upregulation of the contigs; hpf = hours post fertilisation. (B) Pie charts classifying the genes in each cluster into taxonomically restricted genes (blue – no match in the database, unique to *Lobophytum*), Cnidarian-specific (red – matching a database entry in Cnidaria) and other (green – matching a database entry in non-Cnidaria)

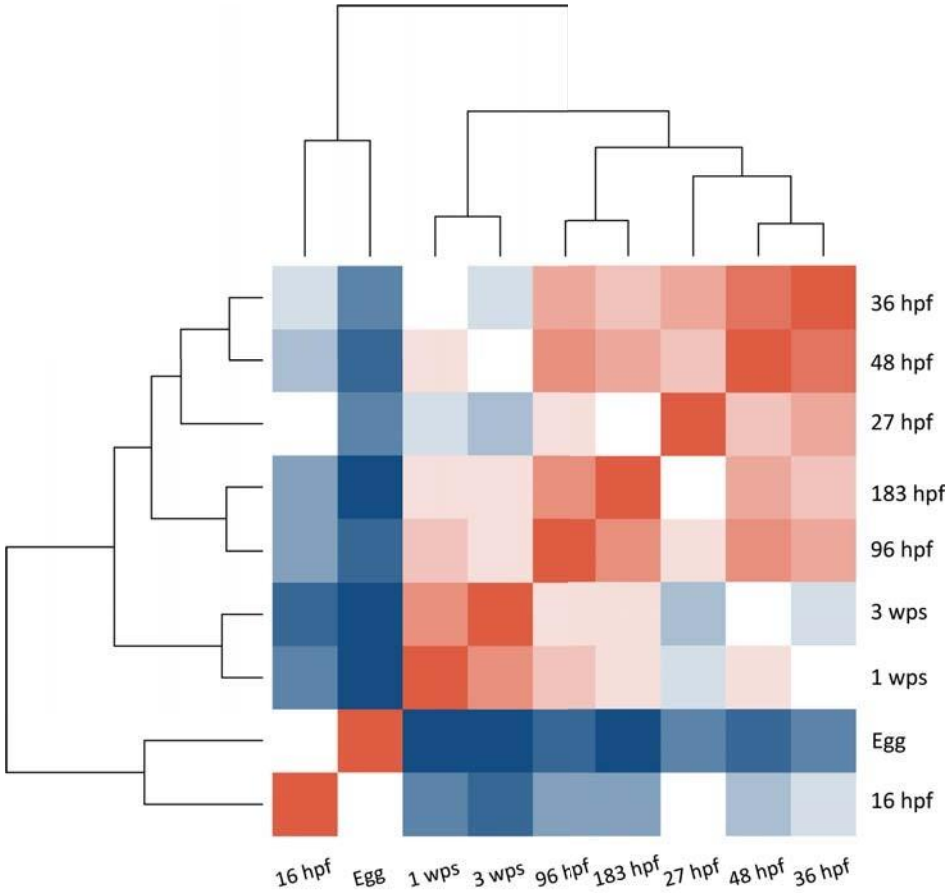


Figure 3.5 Heat map displaying the distance matrix between samples based on expression patterns in Figure 3.4 A. The more intense the red, respectively, the more similar are the expression profiles. Unfert = unfertilised eggs, hpf = hours post fertilisation

As can be seen in the heat map (Figure 3.5), the expression profiles of the unfertilised egg and 16 hpf stages were clearly distinct from those of all other later developmental stages. The early planula stages, 36 and 48 hpf, grouped closely together with the 27 hpf stage. Together with the late planula stages 96 hpf and 183 hpf these 5 stages formed a group with the two polyp stages as closest neighbour (Figure 3.5).

3.4.4 Differential gene expression during settlement and metamorphosis

After observing the formation of sclerites within the first week of settlement, contigs that are specifically upregulated at this stage and at the transition from planula to polyp were investigated in more detail. Comparing differential gene expression of 183 hpf planula, 1 wps polyp and adult to the average expression across all stages showed that 2553 of the upregulated contigs were shared in all 3 stages (Figure 3.6). 278 contigs were specifically upregulated at 183 hpf, whereas 101 contigs were adult specific and 32 polyp specific. Of all 4007 contigs that were upregulated at the polyp stage, the majority were shared either with the planula (n = 667) or adult (n = 755) stages (Figure 3.6). Planula and adult stages shared 14 upregulated contigs that were not upregulated in the polyp stage either.

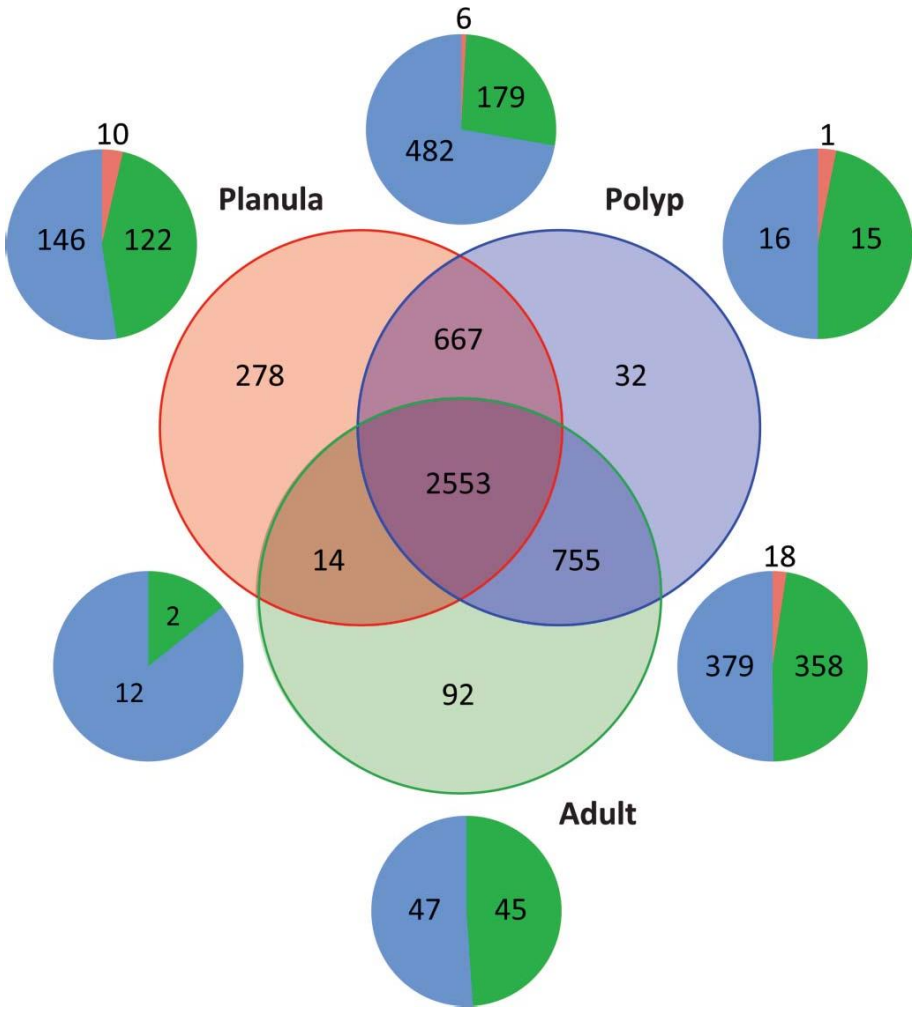


Figure 3.6 Venn diagram comparing upregulated genes in 183 hpf planula, 1 wps polyp and adult when gene expression was compared to the average across all stages. Pie charts classifying the genes in each cluster into taxonomically restricted genes (blue – unique to *Lobophytum*), Cnidarian-specific (red – matching a database entry in Cnidaria) and other (green – matching a database entry in non-Cnidaria)

Closer inspection of the annotation data for the stage specific contigs revealed that the adult–exclusive and planulafadult exclusive sequences did not have hits to Cnidaria–specific sequences (Figure 3.6). The ratio between *Lobophytum*–specific and annotations to common non–Cnidaria was even. Among the adult–exclusive contigs with SwissProt annotation were 5 contigs related to murine sequences describes as caspase 7 (P97864.2, 4 contigs) and 8 (O89110.1, 1 contig). The amino acid sequence of the caspase 7–related sequences was however identical therefore only one sequence (c252346_g1_i4) was used for further analysis. Further, contigs involved in biosynthetic processes with hydrolase activity or cysteine–type endopeptidase activity were highly abundant (Table 3.5). Of the 14 upregulated contigs shared only by planula and adult, 2 contigs had an annotation in the SwissProt database. These contigs were related to the chicken a–subunit of ovomucin sequences (Q98UI9.1). Sequences with a serine–type endopeptidase inhibitor activity were over–represented (Table 3.5).

Of the contigs that were upregulated in adult and polyp stages, 18 were specific to Cnidaria. Among these were 10 contigs matching sequences derived from the staghorn coral *Acropora millepora*. These sequences were associated with proteins found in the organic matrix, such as galaxin (D9IQ16.1, 4 contigs), collagen alpha chain (B8V7R6.1, 2 contigs) and EGF and laminin G domain containing protein (ELP, B8UU78.1, 3 contigs). Among the contigs with a hit against non–Cnidaria were 8 related to carbonic anhydrases (Antarctic icefish CA I, P83299.1, 1 contig; human CA VII, P43166.1, 4 contigs; rat CA II, P27139.2, 3 contigs). In addition 14 contigs were closely related to the extracellular matrix protein, fibrillin 1 and 2 (P35555.3, 8 contigs; P35556.3, 3 contigs; Q61555.2, 3 contigs) from man and mouse. Further among the contigs with SwissProt annotation, sequences for proteins involved in DNA–templated regulation of transcription, actin binding and cell adhesion were over–represented. In addition to these metabolic processes, sequences related to calcium ion binding and immune responses were also highly abundant (Table 3.5).

The only contig upregulated at the polyp stages and unique to Cnidaria was assigned to a luciferin–binding protein (LBP, P05938.1) identified in the sea pansy *Renilla reniformis.* Further, among the contigs with other hits, 8 with hits against human zinc finger proteins (Q5SVZ6.1, 6 contigs; Q8N567.2, 2 contigs) were identified. The molecular function for protein dimerization activity was overrepresented among sequences that were upregulated only at the polyp stage (Table 3.5).

Of the 10 cnidarian–specific contigs differentially expressed at the planula stage, only two were upregulated and these were assigned to the same LBP sequence from *R. reniformis*. A further 8 transcripts related to USOMP 5 (B8VIU6.1) from *A. millepora* were identified

among the contigs with only cnidarian–hits. Other contigs involved in biological processes such as carbohydrate metabolism were highly upregulated. Further, the molecular functions related to calcium ion binding and oxidoreductase activity were over–represented as GO terms (Table 3.5).

In the fraction shared between planula and polyp, one of the upregulated contigs was closely related to the *A. millepora* galaxin and a further three contigs corresponding to the same USOMP5 sequence as identified among the planula specific contigs (above) was also present in the polypƒplanula section. Annotated sequences implicated in ion transport, cellular protein modification processes, lipid biosynthesis and rRNA and tRNA processing were over–represented among the upregulated contigs (Table 3.5).

Among the 2553 contigs that were temporally upregulated in planulae, polyps and adults compared to the average of all stages, 30 cnidarian–only hits and 979 with hits in common non–Cnidaria were identified. The remaining 1544 contigs were *Lobophytum* specific (Appendix Figure 3.2). Among the cnidarian–specific hits, 13 contigs were related to USOMP5 and 3 to galaxin from *A. millepora*. Among the 979 contigs that were annotated to a common non–Cnidaria, 3 were related the human carbonic anhydrase (CA) XIV sequences (Q9ULX7.1) and 4 to the human fibrillin–3 sequence (Q75N90.3). In addition to CAs, 10 contigs were closely related to murine BMP sequences – 6 contigs were annotated as BMP 1 (P98063.2) and 4 as BMP 7 (P23359.2). Besides sequences involved in metabolic processes, regulation of peptide cross–linking as well as cell differentiation are biological processes that were over–represented among the assigned GO terms (Appendix Table 3.1).

All of the *Lobophytum* galaxin–related sequences identified contained signal peptides, acidic domains and dicysteine repeats (Appendix Table 3.2). Further, potential glycosylation sites were identified using but whether these sites are really glycosylated when the protein is translated was not determined. All CA sequences identified among the differentially expressed contigs in the *Lobophytum* transcriptome contained carbonic anhydrase domains and signal peptides (Appendix Table 3.2). Caspase 7–like sequences contained peptidase C14 caspase domain (Appendix Table 3.2). PFAM domain searching also identified a peptidase C14 domain in the caspase 8–like contig, but note that in this case the open reading frame was very short (Appendix Table 3.2).

Table 3.5 Subset of over-represented genes per gene ontology (GO) term identified in uniquely and mutually upregulated contigs in 183 hpf planula (pla), 1 wps polyp (pol) and adult (adu) when temporal expression is compared to average expression of all stages. GO terms are grouped by biological process (BP), cellular component (CC) and molecular function (MF).

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Pla-Adu
BP	Biosynthetic process	-	-	-	-	9	-
BP	Carbohydrate metabolic process	8	-	-	-	-	-
BP	Cell adhesion	-	-	-	17	-	-
BP	Cell communication	-	-	-	7	-	-
BP	Cellular component organization	-	-	-	5	-	-
BP	Cellular protein modification process	-	6	-	-	-	-
BP	G–protein coupled receptor signalling pathway	-	-	-	17	-	-
BP	Immune response	4	-	-	7	-	-
BP	Ion transport	-	9	-	-	-	-
BP	Lipid biosynthetic process	-	5	-	-	-	-
BP	Metabolic process	9	-	-	-	-	-
BP	Methylation	-	2	-	-	-	-
BP	Nodulation	-	2	-	-	-	-
BP	Nucleoside diphosphate phosphorylation	4	-	-	-	-	-
BP	Oligosaccharide biosynthetic process	-	2	-	-	-	-
BP	Phospholipid catabolic process	-	4	-	-	-	-
BP	Protein folding	3	-	-	-	-	-
BP	Proteolysis	-	-	-	16	5	-
BP	Regulation of cell growth	-	4	-	-	-	-
BP	DNA–templated regulation of transcription	-	-	-	19	-	-
BP	rRNA modification	-	2	-	-	-	-
BP	rRNA processing	-	3	-	-	-	-
BP	Steroid hormone mediated signalling pathway	-	-	-	4	-	-
BP	tRNA processing	-	5	-	-	-	-
CC	Cytoskeleton	-	-	-	15	-	-

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Pla-Adu
CC	Extracellular matrix	-	-	-	4	-	-
CC	Extracellular region	-	7	-	-	-	-
CC	Integral component of membrane	-	-	-	45	-	-
CC	Membrane	15	-	-	26	-	-
CC	Proteinaceous extracellular matrix	-	-	-	14	-	-
MF	Actin binding	-	-	-	8	-	-
MF	Ammonia−lyase activity	-	-	-	-	9	-
MF	Calcium ion binding	14	-	-	40	-	-
MF	Calcium−dependent phospholipid binding	3	-	-	-	-	-
MF	Cysteine−type endopeptidase activity	-	-	-	-	5	-
MF	DNA binding	-	-	-	22	5	-
MF	G−protein coupled receptor activity	-	-	-	17	-	-
MF	GTP binding	-	-	-	-	4	-
MF	GTPase activity	4	-	-	-	-	-
MF	Heparan sulfate proteoglycan binding	-	-	-	8	-	-
MF	Hormone activity	-	3	-	-	-	-
MF	hydrolase activity	-	10	-	-	5	-
MF	Insulin−like growth factor binding	-	4	-	-	-	-
MF	Ion channel activity	-	8	-	-	-	-
MF	Kinase activity	-	-	-	4	-	-
MF	Metal ion binding	-	13	-	-	-	-
MF	Methyltransferase activity	-	8	-	-	-	-
MF	N−acetyltransferase activity	-	-	-	-	3	-
MF	Nucleic acid binding	-	-	-	-	5	-
MF	Nucleoside diphosphate kinase activity	4	-	-	-	-	-
MF	O−methyltransferase activity	-	3	-	-	-	-
MF	Oxidoreductase activity	11	4	-	-	-	-
MF	Phospholipase activity	-	4	-	-	-	-

3.5 DISCUSSION

3.5.1 Developmental time line for *Lobophytum pauciflorum*

After successful collection and fertilisation of soft coral gametes, the development of *L. pauciflorum* was studied over a period of 3 weeks. As in the case of *Lobophytum pauciflorum* reported here, irregular morphology following fertilisation and during the first hours of development has also been described for *Alcyonium digitatum* (Matthews, 1917) and various time points of first cleavage resulting in 2 or more cells was observed in *Renilla reniformis* (Wilson, 1883). Further, in both *Dendronephthya hemprichi* and *Clavularia hamra* from the Red Sea, mixed cleavage patterns and 'bizarre' shapes were also observed together with superficial cleavage and segmentation (Benayahu, 1989; Dahan and Benayahu, 1998). The high yolk content in octocoral oocytes might explain the tendency towards superficial cleavage (Mergner, 1971) and the precedents mentioned above suggest that mixed cleavage patterns are common in development across the Alcyonacea. In the starlet sea anemone *Nematostella vectensis*, a variety of different early cleavage patterns have been observed, and the 2−nuclei stage is almost indistinguishable from egg, as no cytokinesis occurred (Fritzenwanker *et al.*, 2007). The authors further observed fusion between 2−cell and 4−cell as well as between 4−cell and 8−cell stage embryos. As is likely to be the case in *L. pauciflorum*, cleavage in *N. vectensis* is holoblastic, starting with the third cell cycle (Fritzenwanker *et al.*, 2007). The lack of obvious early division stages in *L. pauciflorum* development and irregular morphology may therefore not be so unusual, but rather an ancestral pattern within Anthozoa common to sea anemones and octocorals. Close observation of fertilised oocytes with regular sampling, e.g. every 30 min until signs for first cleavage occur, should be carried out in the future. Such a time line could elucidate whether nuclear division occurs prior to cytosolic division, resulting in a potential formation of a syncytium.

A few hours after fertilisation, *Lobophytum* embryos developed into swimming planulae that eventually settled and underwent metamorphosis in a completely asynchronous manner, making determination of the time window of competency and the task of describing the settlement and metamorphosis processes very difficult. Planulae of *L. crassum* were observed to be competent to settle for at least 20 days (Uehara *et al.*, 1987). In the case of *D. hemprichi*, some planulae were observed to settle and metamorphose at 2 days post−fertilisation, but others from the same batch did so over the following 3 − 4 days (Dahan and Benayahu, 1998). Moreover, *D. hemprichi* planulae appear to be highly longevic and remained competent to settle for 65 days. However, despite the maximum larval longevity of 100 days, 50 % of the *D. hemprichi* planulae had died after 70 − 75 days (Dahan and Benayahu, 1998),

and the long competency of *D. hemprichi* was a consequence of inhibiting metamorphosis by applying antibiotics to the filtered seawater (Dahan and Benayahu, 1998). In the present case, however, *L. pauciflorum* planulae were maintained without antibiotics and might therefore be competent to settle for an extended period of time. As larvae were competent to settle after 4 days of development, as observed for this species in 2015 and in the congeneric species, *L. crassum* (Uehara *et al.*, 1987), it is likely that the cue to induce settlement in *L. pauciflorum* remains to be found. Neuropeptides of the LW−amide family reliably induce metamorphosis in other anthozoans including *Acropora* spp. (Iwao *et al.*, 2002; Erwin and Szmant, 2010) and hydrozoans, e.g. *Hydractinia echinata* (reviewed in Müller and Leitz, 2002). The data suggest a requirement for a settlement cue in soft corals; CsCl appears to not be an appropriate proxy for such a cue, and the literature suggests that LW−amides are candidates. However LW−amide peptides are highly taxon specific and the appropriate sequence(s) for *L. pauciflorum* remain to be determined.

The approximate timeline for settlement and development of mesenteries in *L. pauciflorum* appears to be in line with what has been reported for *L. crassum* (Uehara *et al.*, 1987), *Alcyonium digitatum* (Matthews, 1917) and *Dendronephthya hemprichi* (Dahan and Benayahu, 1998), despite the lack of consistent temperature conditions across these studies. In externally developing animals, early development is typically temperature sensitive, embryos developing faster at higher water temperatures and slower at cooler temperatures (Dahan and Benayahu, 1998). However, temperatures above a taxon specific threshold can induce deformation and other abnormalities (Wilson and Harrison, 1997).

Although at a morphological level, phylogentically diverse soft corals develop in similar ways, molecular data may reveal cryptic developmental diversity and will be essential in terms of the broader evolutionary interpretation. Okubo and colleagues (2016) were able to link similar morphological shapes with a similar patterns of expression of othologous genes in different hard corals species, highlighting the conservation of important patterns in hard coral development. The molecular data reported here for *Lobophytum* provide the first step in understanding soft coral development at the molecular level.

3.5.2 Transcriptome assembly

The *L. pauciflorum* transcriptome reported here was assembled from a large number of genotypes as, in addition to two adult colonies, the developmental stages used for RNAseq library construction were generated from 8 different female and 4 different male colonies. As the adult colonies used contained *Symbiodinium*, sequences that were derived from the zooxanthellae were discarded prior to further analysis. The number of contigs assigned as *Symbiodinium* derived was 76126; by comparison, transcriptome sequencing of *Symbiodinium* sp. from clade A and B resulted in 56000 assembled sequences (Bayer *et al.*, 2012). Although the number *Symbiodinium* sp. sequences recovered with bioinformatics means was higher than the assembled sequences from Bayer and colleagues (2012), the assembly of *Symbiodinium* sp sequences yielded 72152 and 76284 from clade A and clade B, respectively, prior to clustering with a sequence identity of 90 %. The numbers of assembled sequences from Bayer and colleagues prior to clustering were therefore similar to the sequences recovered from the *Lobophytum* transcriptome assembly, which was not clustered.

The much larger number of assembled contigs in the *L. pauciflorum* host fraction (359799 contigs) compared to the recently published transcriptomes of *Corallium rubrum* (48074 contigs) (Pratlong *et al.*, 2015) and of *Gorgonia ventalina* (90230 contigs) (Burge *et al.*, 2013) is presumably due in part to the large number of genotypes that were sequenced, i.e. it is possible that alleles have been mis−assigned as distinct “genes”. By comparison, the assembled transcriptomes of the gorgonians *Leptogorgia sarmentosa, Eunicella cavolinii* and *E. verrucosa* contain between 13300 and 20730 contigs (Romiguier *et al.*, 2014). However, all 3 of these studies used clustering algorithms such as CD−HIT−EST andfor more stringent criteria to discard assembled sequences (only open reading frames (ORFs) of at least 200 bp were considered) (Romiguier *et al.*, 2014; Pratlong *et al.*, 2015). In the present study, the sequences assembled by Trinity were not clustered based on similarity prior to mapping, as Corset was used for clustering of the mapped read counts afterwards. A preliminary genome assembly of *Lobophytum pauciflorum* based on genomic DNA extracted from sperm from a single individual is now available, and predictions from this suggest that it contains approximately 30000 protein coding genes (C. Shinzato, pers. communication). 36 % of the *C. rubrum* and 44 % of the *G. ventalina* transcriptome could be annotated using similar databases as in this study (Burge *et al.*, 2013; Pratlong *et al.*, 2015). In the present case, using the longest annotated isoforms of transcripts resulted in approximately 33 % annotation (Table 3.2), which is in the same range as the published estimates for the two other octocorals. However, when only the longest isoforms of each transcript were considered, coverage was only 20 % (Table 3.2),

suggesting that some contigs may not be assembled correctly. Therefore selection of contigs based on the longest ORF might be a useful approach to improve annotation of the present transcriptome.

3.5.3 Temporal gene expression patterns compared to adults

Using a cut–off at FDR < 0.05 and an absolute fold change of at least 2, approximately 1.5 % of the transcripts were differentially expressed throughout early development relative to adults. The majority of these contigs were expressed at lower levels in developmental stages relative to adults. A variety of developmental studies using the staghorn coral *A. millepora* showed detection of differentially expressed genes is highly variable. Whereas Grasso and colleagues (2008) detected 1084 of 5081 unique peptide coding genes (21 %) on their microarray that were differentially expressed between 2 consecutive developmental stages, a later study showed that only 2 % of a microarray containing > 8000 unique *Acropora* sequences were differentially expressed when settlement and metamorphosis were induced using CCA extract (Grasso *et al.*, 2011). The identities and relatively small numbers of genes whose expression changes during the development of *Lobophytum* are consistent with data for *Acropora* (Grasso *et al.*, 2011) and suggest that genes involved in embryogenesis were already specifiedƒdifferentiated in the last common ancestor before the divergence of Hexacorallia and Octocorallia.

Over–representation of GO terms involved in hedgehog receptor activity in expression group II (Table 3.4) is linked to abundant expression of contigs related to dispatched homologs. As part of the hedgehog signalling pathway, dispatched encodes a membrane protein that transmits the hedgehog signal from hedgehog expressing cells (Burke *et al.*, 1999). The *N. vectensis* dispatched gene has been identified, but its expression pattern throughout development has not yet been reported (Walton *et al.*, 2006; Matus *et al.*, 2008). In the present study, hedgehog related sequences were not differentially expressed, however in *N. vectensis* hedgehog expression is co–localised with that of Wnt (Kusserow *et al.*, 2005; Adamska *et al.*, 2007). Co–expression of dispatched– and Wnt–like sequences in *Lobophytum* suggests that hedgehog may also be expressed at a similar time in *L. pauciflorum*, but at levels below the threshold for detection. *Lobophytum* sequences matching well to hexacorallian Wnt 1 sequences (Genbank NR database) were more abundant in unfertilised eggs than in adults but were not more abundant in other developmental stages, suggesting that these sequences are provided maternally. Maternal transmission has been shown for Wnt3 genes in *Clytia hemisphaerica* (Momose *et al.*, 2008). Higher abundance of dispatched–like sequences in unfertilised eggs in *L. pauciflorum* suggests that these sequences are maternally provided. The apparent diversity of expression patterns observed suggests that a detailed *in–situ* hybridisation examination of Wnt and hedgehog ligands and pathway components is warranted.

Some sequences that are more abundant in the planula stage compared to adults (G III, Figure 3.4, Table 3.4) were involved in cell growth and RNA–DNA hybrid ribonuclease activity. The abundance of these sequences began to increase after 16 h of development and continued to increase until 183 hpf. In *A. millepora*, sequences involved in replication and RNA processing were highly abundant during the prawn–chip (pre–gastrulation) stage (Grasso *et al.*, 2008). In Bilateria, the mid–blastula transition is thought to generally correspond to the point of activation of the zygotic genome (Newport and Kirschner, 1982a, b; Wieschaus, 1996) though some zygotic genes might be expressed beforehand (Mathavan *et al.*, 2005). In addition to upregulation of sequences involved in RNA–DNA hybridisation and cell growth, the expression profiles at 16 hpf being more similar to unfertilised eggs and at 27 hpf more similar to the planula stage (Figure 3.5) implies that zygotic gene expression is initiated between 16 hpf and 27 hpf. Although the morphology of an embryo at 27 hpf is very different from that of a non–swimming planula at 36 hpf, comparison of differential expression levels of these two stages showed that at 27 hpf the embryo already possesses most of the molecular tool kit that an embryo needs to develop into a planula. Gene expression starts before morphological changes occur, as expressed genes frequently require post–transcriptional and post–translational modifications (Storey and Storey, 2004). Thus, taking over–representation of GO terms and similaritiesƒdifferences in expression profiles together, it is likely that gastrulation occurs during the 16 – 27 hpf interval in *Lobophytum*. Studying differential expression compared to adult expression in *L. pauciflorum* might hinder detection of key players in embryogenesis but also during adult stages. Therefore, future developmental studies of *L. pauciflorum* development embryos at 16 could be used as a baseline for differential gene expression to determine pre– and post–gastrulation expression patterns and key players during these developmental phases. However, the mode of gastrulation, i.e. via delamination or ingression has yet to be identified. This could be done, for example, by a more thorough sampling during this time period and histological analysis.

The proportion of *Lobophytum* specific sequences, those only identified in other Cnidaria and commonly found in other non–Cnidaria varied between expression clusters (Figure 3.4 B). In *A. millepora*, *Acropora*–specific genes were always of lowest abundance among differentially expressed genes in each expression cluster (Grasso *et al.*, 2008). However, this experiment was based on microarray analysis and rather than transcriptomics. Whereas few cnidarian–unique genes were differentially expressed throughout development in the Grasso study, soft coral specific contigs were highly abundant in groups I, III and VII, which were most highly expressed during the planula and polyp phases, the planula phase and the polyp stage, respectively. Further, the highest proportion of ‘core’ genes, defined as genes

represented in animals and other kingdoms, were observed amongst sequences upregulated during the planula, planula–polyp and primary polyp stages (Grasso *et al.*, 2008). In *L. pauciflorum,* a similar pattern emerged, contigs in expression groups V and VI corresponding to 'core' genes being more highly represented during the planula and polyp stages (Figure 3.4 B). This could potentially indicate that common cellular pathways were activated throughout later development whereas more specific developmental pathways are employed at earlier time points. Due to the different techniques and thresholds applied, the low annotation of the soft coral transcriptome could artificially increase the proportion of *Lobophytum* specific genes. Therefore interpretation of these proportions should be considered carefully.

3.5.4 Differential gene expression during settlement and metamorphosis

Sclerites were clearly visible within the tissue of *L. pauciflorum* polyps within a week of settlement (Figure 3.2), prompting a more extensive investigation of the differential expression of potential biomineralisation genes in the late (183 hpf) planula, 1 wps polyp and adult stages. The vast majority of contigs at higher levels in these stages relative to the averaged expression across stages were shared between the three target stages. Amongst these (n=2553), were soft coral homologs of two genes previously implicated in scleractinian calcification – the uncharacterised skeletal organic matrix protein SOMP5 and galaxin (Reyes–Bermudez *et al.*, 2009; Moya *et al.*, 2012). In addition to these cnidarian–specific genes, other genes with potential roles in calcification were also identified, including several carbonic anhydrases – genes long known to play important roles in calcification in hard corals (Hayes and Goreau, 1977) and many bilaterians. Three *Lobophytum* sequences related to human CA XIV were upregulated in the planula, polyp and adult stages. This human enzyme is a membrane–bound CA (Mori *et al.*, 1999). In polyps and adults, but not in planulae, contigs related to bilaterian sequences CA I, II and VII were also upregulated. In contrast to CA XIV, CA I, II and VII are cytosolic proteins (Lowe *et al.*, 1990; Hewett–Emmett and Tashian, 1996) Alignment of the CA sequences identified in this study with known soft coral and hard coral sequences revealed the best matches for the *Lobophytum* CAXIV–like sequences to be *N. vectensis* A6QR78 (Jackson *et al.*, 2007) and *Stylophora pistillata* B5SU02 (Jackson *et al.*, 2007; Moya *et al.*, 2008), which fell into a large clade of invertebrate secreted and membrane–bound CAs in phylogenetic analysis (Moya *et al.*, 2008). In *L. pauciflorum* the potentially membrane–bound CA was also expressed in pre–settlement stages suggesting that membrane–bound CAs might play important roles during metamorphosis and onset of calcification (de Boer *et al.*, 2006). The *Lobophytum* sequences c233399_g1_i2 and c254563_g1_i1 showed sequence similarity to a *S. pistillata* CA described as CAII–like, suggesting that this might indeed be a cytosolic CA (C0IX24, Bertucci *et al.*, 2011)(Appendix Figure 3.3). While membrane–bound CAs were upregulated pre– and post–settlement in *Lobophytum*, cytosolic CAs were upregulated only in post–settlement stages, suggesting that cytosolic CAs are more important in post–settlement development than prior to metamorphosis in this species.

Alignment of the three soft coral galaxin contigs with sequences from *A. millepora and G. fasicularis* (Appendix Figure 3.4) indicated that the *Lobophytum* sequences had greater similarity to *Amgalaxin–like 1* and *2* than to the adult type galaxins from hard corals. *Lpgalaxin-like*(c216859_g1_i1) was upregulated during the planula, polyp and adult stage, whereas *Lpgalaxin–like 2* and *3* (c238372_g1 and c209119_g1) sequences were upregulated

during polyp and adult stages and planula and polyp stages, respectively. In *A. millepora,* expression of galaxin–like sequences was exclusively associated with the early stages of calcification (Reyes–Bermudez *et al.*, 2009), as reported here for the soft coral. While *Amgalaxin–like 1* and *2* were expressed in pre–settlement planula and early primary polyps but not in adults, *Amgalaxin* was almost exclusively expressed in adults (Reyes–Bermudez *et al.*, 2009). A similar pattern emerged in *Galaxea fasicularis,* where the galaxin protein was only found in adults but not in planulae (Fukuda *et al.*, 2003). The fact that galaxin–like sequences are expressed at similar points in development, at around the initiation of calcification, in hard and soft corals suggests that, contrary to expectations, at least some of the mechanisms underlying the deposition of aragonite and calcite in the Cnidaria may have a common evolutionary origin. Clearly a more extensive investigation of the expression patterns of soft coral galaxin genes, including in situ hybridisation experiments, should be a priority for further investigation.

Caspases are key components of apoptotic cell death pathways and are essential for normal embryogenesis, immunological defence and both cellular and tissue homeostasis across the Metazoa. Acute and prolonged exposure of *A. millepora* juveniles to elevated CO_2 led to upregulation of several caspases (Moya *et al.*, 2016). The exclusive upregulation of caspases observed in adult *Lobophytum* could be indicative of a stress response, or that tissue remodelling was occurring. The sequences exclusively upregulated at the adult stage were assigned to two different murine caspase sequences in the SwissProt database, however the amino acid sequence of the caspase 8–like contig was shorter though identical to the caspase 7–like contig. Comparison to other cnidarian caspase sequences revealed that both soft coral sequences were most similar to each other but also to the FAS associated death domain (FADD) protein identified in *A. millepora* (A0A0B5KML6, Sakamaki *et al.*, 2014) and *Hydractinia echinata* caspase–3 (B1GXL8.1, Wittig *et al.*, 2011) but also *A. millepora* caspase–8–like sequences (A0A0B5KPL7, A0A0B5KML6, Sakamaki *et al.*, 2014)(Appendix Figure 3.5). The close similarity of the soft coral sequences suggests that both contigs encode the same protein, matching well to caspase–3ƒ7 like sequences (Genbank NR database). In *H. echinata* metamorphosis depends on caspase–3 activity and knockdown of caspase–3 highlighted the importance of apoptosis especially at the anterior pole of the metamorphosing larvae (Seipp *et al.*, 2007; Wittig *et al.*, 2011). Expression of caspase–like sequences in *L. pauciflorum* was only upregulated in fully differentiated adults, suggesting that these isoforms are not involved in metamorphosis but rather during later stages for example tissue differentiation.

The ovomucin–related sequence was only retrieved among the contigs upregulated during the late planula stage. Ovomucin is an insoluble glycoprotein, responsible for gel like properties of egg white with an extracellular location first identified in the thick part of the egg white (Robinson and Monsey, 1971). Glycoproteins of different macromolecular fractions but similar structure of component proteins and polysaccharides were identified in coral reef cnidarians and termed 'mucin' (Ducklow and Mitchell, 1979). After secretion, Ducklow and Mitchell (1979) observed structural changes under the scanning electron microscope from liquid mucus to mucus floc or web material. For *A. formosa* mucin as principal component is responsible for the viscoelastic properties of the mucus (Meikle *et al.*, 1987). Considering the structural and viscoelastic properties of cnidarian mucins (Ducklow and Mitchell, 1979; Meikle *et al.*, 1987) the expression of an ovomucin–related sequence in late planulae suggests that it might play a role during the settlement process in *Lobophytum*. Secretion of mucus could facilitate attachment of the swimming planula to a suitable substrate. On the other hand, an immunological role has been proposed for coral mucins (Hayes, 1998). An activation of the coral innate immune system was also suggested by the over–representation of sequences related to immune response (Table 3.5). However, an onset of immunity does not exclude beneficial properties supporting settlement. It rather supports the idea that the late planulae are competent to settle at this stage of development. Nevertheless a more thorough analysis of the ovomucin–like sequences is needed to draw further conclusions about homology and its potential function.

For a better identification of key players in settlement and metamorphosis, sequence data from primary polyps would be desirable. Due to the asynchronous settlement only few primary polyps and very low RNA yields, cDNA library preparation for this time point could not be performed. Once a more suitable settlement cue is identified, this problem will be resolved and will allow a better resolution of the settlement and metamorphosis process in *L. pauciflorum*.

3.6 CONCLUSIONS

This is the first study of any octocoral to combine a morphological description of early development with molecular investigation of the underlying mechanisms. At the morphological level, the early development of *Lobophytum* was consistent with what has previously been described for other broadcast spawning Alcyonacea, suggesting that common pathways of early development with a wide phylogenetic basis in this group. On the other hand, gastrulation in *Lobophytum* (and perhaps other octocorals) is likely to differ fundamentally to what has been described in the hexacorallians *A. millepora* and *N. vectensis*. Based on differential expression data, it is likely that zygotic gene expression is initiated between 16 hpf and 27 hpf, and that gastrulation occurs in this time window. Comparing expression data across the planulaʄpolypʄadult spectrum of stages allowed identification of candidate genes for roles in soft coral calcification, and highlighted that, as in *Acropora*, competent *Lobophytum* planulae express not only the molecular tool kit for calcification but also that required for an immune response.

This study provides the baseline for future studies on development in *Lobophytum pauciflorum* focusing on first cell division and gastrulation. Sampling over a narrow time window leading up to the first cell divisions, e.g. every 30 min after fertilisation until the first blastomeres are observed, followed by histological analysis using nuclear staining, could clarify whether nuclear divisions occur prior to cytosolic divisions. It is to be hoped that histological sectioning of embryos during the time window identified will clarify the mode of gastrulation in *L. pauciflorum*. To better understand early development, a more intensive sampling regime is desirable, e.g. sampling every hour from 14 hpf to 30 hpf. A genome guided transcriptome assembly or a more stringent reassessment of the herein described transcriptome assembly, e.g. considering contigs with an ORF longer than 200 bp, might be useful to reduce transcript isoforms and to attain a more concise transcriptome assembly. More comparative work will assess completeness of this transcriptome and further reveal *Lobophytum* specific characteristics. However, this is not only the first transcriptome for soft coral of the Alcyoniidae family but also the first RNAseq analysis of developmental gene expression for a soft coral species, and hopefully marks beginning of a new era in the study of soft coral development.

Chapter 4 The microbiome of the soft coral *Lobophytum pauciflorum* and its response to environmental stress

This chapter is more detailed than the draft version of the corresponding paper, which will be submitted to FEMS. The paper draft for submission is attached in the Appendix for this chapter (Appendix Chapter 4).

Wessels W, Sprungala S, Watson SA, Miller DJ, Bourne DG (to be submitted) The microbiome of the soft coral *Lobophytum pauciflorum* and its response to environmental stress.

All sampling and sample processing was done by W Wessels. SA Watson assisted with experiment design and seawater chemistry analysis. DG Bourne helped with the sequence analysis of the microbiome data. W Wessels analysed the data and wrote the chapter and manuscript after intellectual contributions by all co−authors.

4.1 ABSTRACT

Bacteria associated with marine invertebrates are increasingly recognised as having important functional roles benefiting the host including production of antibacterial products that exclude other harmful microorganisms and recycling of essential nutrients. While the microbiomes of scleractinian corals have been well investigated, octocorals of the order Alcyonacea have received less attention. This study characterised the microbiome of a gonochoric octocoral, *Lobophytum pauciflorum*, and investigated if sex specific differences and environmental stresses influence the diversity and stability of the associated microbiome through amplicon profiling of the bacterial 16S rRNA gene. The core microbiome of *L. pauciflorum* was dominated by sequences affiliated to Spirochaetes and y−Proteobacteria, representing 43 % and 27 % of the community, respectively. Within the y−Proteobacteria, *Endozoicomonaceae* related sequences were dominant, representing 21% of retrieved sequences. Among the dominant class affiliations, no sex−specific differences were detected though a 2−fold higher relative abundance of unassigned sequences was found among female samples than males. These potentially novel sequences contributed to observed differences between sexes as detected by a multivariate analysis at OTU level and therefore offer the chance to discover yet undescribed bacterial strains from this novel environment. Exposing the *Lobophytum pauciflorum* fragments to increased temperature (31 ˚C), decreased pH (7.9) and a combination of increased temperature and decreased pH for 12 days did not alter the microbial community indicating that the dominant community members of the soft coral microbiome are stable and resilient to short term environmental stress.

4.2 INTRODUCTION

Tropical coral reefs are among the most diverse ecosystems on earth with hard corals (order Scleractinia) and soft corals (order Octocorallia) representing two of the major benthic communities constituting these ecosystems (e.g. Done, 1982). The majority of reef−building corals are hard corals (Veron, 1986) although some soft coral species consolidate their reef with sclerites (Schuhmacher, 1997; Jeng *et al.*, 2011). Despite lacking an exoskeleton, soft corals provide additional structural complexity for fishes and other marine invertebrates, though their importance for reef ecosystems is often overlooked. Soft coral have a well−developed chemical defence mechanism, which has been the focus of much of the research into these reef inhabitants (Tursch *et al.*, 1974; Coll *et al.*, 1982; Coll *et al.*, 1986; Coll *et al.*, 1989; Coll *et al.*, 1995).

The soft coral *Lobophytum pauciflorum* (Ehrenberg, 1834) is a common representative of the shallow waters in the Indo−West Pacific (Tursch and Tursch, 1982; Verseveldt, 1983; Benayahu, 2002). Like most soft corals (Kahng *et al.*, 2011), *L. pauciflorum* is a gonochoric species with dimorphic polyps, though the importance of separate sexes in this species is yet to be determined. Interestingly differences in the UV protective mycosporine−like amino acids (Michalek−Wagner, 2001) and complimentary secondary metabolite concentration (Fleury *et al.*, 2006) identified between female and male colonies, indicate the possibility for physiological differences between the sexes. Secondary metabolites were found to have cytotoxic and antimicrobial properties in this species (Zhao *et al.*, 2013), though physiological differences in secondary metabolites between different sexes have not been investigated. Sex determining mechanisms or further discrimating factors consolidating the hypothesis of the origin of the physiological differences observed, are so far unknown. If physiological differences between *L. pauciflorum* sexes exist however, they might be driven by a differentially regulated immune system. This phenomenon has been observed in bilateria, where sex−specific differences in the immune system have been identified as one possible source for physiological differences (Klein, 2000; Peng *et al.*, 2005; Love *et al.*, 2008; Nunn *et al.*, 2009). The immune system may also influence the associated microbial communities of the soft coral with an increasing number of studies recognising the importance of microbial constitutents within the coral organsim, the sum of which is termed the coral holobiont (Bosch and McFall−Ngai, 2011). These microbial communities have been well studied in hard corals, however, despite the abundance of the soft coral *L. pauciflorum*, this species has been little

studied at either the organismal, molecular or microbial levels (Fan *et al.*, 2005; Yan *et al.*, 2010a; Yan *et al.*, 2010b; Yan *et al.*, 2011).

The cnidarian immune system contains homologous features to those identified in the bilaterian innate immune system (Lesser *et al.*, 2004; Palmer *et al.*, 2012). Some of those shared components are pattern recognition molecules and signal transduction pathways (Lesser *et al.*, 2004; Augustin *et al.*, 2010; Palmer *et al.*, 2012). Upon exposure to a common fungal parasite, the immune system of the Caribbean sea fan *Gorgonia ventalina* responds with differential expression of pattern recognition molecules and antimicrobial peptides amongst others (Burge *et al.*, 2013). In order to inhibit proliferation of bacteria, removal of infected cells via the apoptotic pathway is crucial. Host induction of apoptosis is the preferred mechanism for terminating the immune response following control of infection because tissue damage, an inevitable consequence of inflammation, is limited with this mode of cell death (Haslett, 1999; Kanaly *et al.*, 1999). In all metazoans, apoptosis is essential for embryonic development, immunological defence, oxidative stress and cellular as well as tissue homeostasis. In the coral relative *Hydra*, apoptosis is morphologically indistinguishable from apoptosis mediated cell death in vertebrates and invertebrates (Cikala *et al.*, 1999). In addition to the morphological similarities, two genes with strong homology to caspase−3 are expressed and the caspase−3 like enzyme is active during apoptotic cell death in *Hydra* (Cikala *et al.*, 1999). The recent description and characterisation of caspases in *Acropora millepora* (Moya *et al.*, 2016) strengthens the broad apoptotic cell death repertoire in the common ancestor for Cnidaria and Bilateria.

The term microbiome describes 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that share the body space' (Lederberg and Mccray, 2001). Scleractinia corals benefit from the microbial associates in terms of nitrogen fixation (Rohwer *et al.*, 2002; Lesser *et al.*, 2004), antibiotics production (Ritchie, 2006) and mucus recycling and food supply (Wild *et al.*, 2004). Within hard corals, studies have identified highly diverse though often conserved microbial populations associated with coral species (Rohwer and Kelley, 2004; Bourne *et al.*, 2008). During periods of environmental stress the microbiome of corals has been shown to shift either driven by a host response andfor changes in the microbiome itself (Bourne *et al.*, 2008). For example during bleaching events the coral associated microbiomes are more diverse (Bourne *et al.*, 2008) accompanied by a shift in functional composition from autotrophic to heterotrophic communities (Littman *et al.*, 2011). Vega Thurber and colleagues (2009) observed shifts from healthy−associated coral microbial community to a community often found in diseased corals under experimental stress in *Porites*

compressa. Shifts in microbial community away from normal homeostasis might also trigger the innate immune response in order to fend off potential infections. Recently studies have also begun to explore the core microbiome, a subset of microorganisms that is shared by most individuals of the species with crucial functional roles, though not necessarily composed of the most abundant microorganisms functions (Ainsworth *et al.*, 2015). These communities may play a vital role in coral health during time of stress with loss of the core microbiome contributing to reduced host fitness.

The microbiome of gorgonians has been investigated previously and identified members of the γ–Proteobacteria class were the dominant members of the associated microbiomes. The majority of retrieved sequences from 16S rRNA gene profiling studies were identified as affiliated with the genus *Endozoicomonas* (Sunagawa *et al.*, 2010; Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). This host–microbial association is potentially symbiotic due to evidence pointing at a long–term partnership with little geographic or seasonal influence (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). Further, this association seems to be species–specific with each gorgonian species having a host specific *Endozoicomonas* ribotype even on a regional scale and potential host–dependant selection factors (La Rivière *et al.*, 2015). The coral relative *Hydra* has previously been established as model organism for cnidarian microbiomics. The adult microbiome is established within the first weeks of development and stable throughout time (Franzenburg *et al.*, 2013). After 30 years in culture, *Hydra* microbiomes in culture were still the same as the ones from polyps in the original pond and significantly different between species that have been in culture (Fraune and Bosch, 2007; Fraune *et al.*, 2009). The *Hydra* microbiome was dominated by Proteobacteria and Bacteroidetes affiliated organisms, with microbial partnerships being specific to each species (Fraune and Bosch, 2007) and the closer the species are related to each other the more similar the associated microbial community was (Bosch, 2013).

In this study we investigated the microbiome of the soft coral *Lobophytum pauciflorum* and explored the potential to discriminate between male and female colonies on the microbial level. Further we investigated the effect of experimentally applied natural stressors such as elevated temperature and CO_2 to determine the acute response (1 d) of *L. pauciflorum* as well as its ability to deal with these stressors for a few days (12 d). We studied caspase activity as part of the apoptotic immune response in the coral host and the effect of both temperature and CO_2 on the microbial community profiles of *L. pauciflorum*.

4.3 MATERIALS & METHODS

4.3.1 Animal collection and maintenance

Two separate collections of *Lobophytum pauciflorum* colonies were undertaken in August 2013 and October 2014 from around Orpheus and Pelorus Islands (18.57˚ S, 146.48˚ E; 18.54˚ S, 146.49˚ E according to the GBRMPA permit G12ƒ35295.1), to supply samples for the experimental analyses. For the sex–specific microbiome analyses, 19 *L. pauciflorum* colonies were brought to Orpheus Island Research Station (OIRS) and held in a 1000 L flow through tank and acclimated for one week prior to sampling of two replicate lobes from each colony. One lobe was immediately snap frozen for total DNA extraction and the second lobe transferred into HEPES buffered seawater with 4 % formaldehyde for fixation. After taking tissue samples the colonies were returned to the ocean to a marked location, before bringing them back to the station one month later with the sex of the colonies determined by histology andfor coral spawning.

For the temperature and CO_2 stress experiments, fragments of 6 *Lobophytum pauciflorum* colonies were transported and cultured in a 1000L tank with flow–through seawater at the Marine Aquaculture Research Facility Unit at James Cook University, Townsville for further propagation. After one week of acclimation the fragments were cut into two pieces for the first time followed by a recovery period of 3 days. The fragments were halved again so that 4 small fragments in total of each colony were obtained, which were transported back to OIRS and left to acclimate for 2 weeks prior to experimental manipulation.

4.3.2 Histological preparation and sex identification

Following fixation for 24 h, tissue samples were washed twice with tap water and decalcified overnight in 10 % formic acid. After decalcification samples remaining formic acid was rinsed off and samples were cut for transactional and longitudinal analyses. Until further processing, samples were stored in 70 % ethanol. Prior to sectioning, the tissue samples were embedded in paraffin. Three sections every 50 µm with a thickness of 5 µm were prepared for each sample. Sections were stained with hematoxylin and eosin to facilitate gonad recognition. Gonads were identified using a 10x magnification of a compound stereomicroscope (Olympus). Presence of oocytes and spermatogonia indicated the sex of the colony as female or male, respectively. If gonads were not clearly visible in tissue sections, the sex of the colony was identified during the annual coral spawning event. In total the sex of 17 colonies was

identified: 8 colonies were females and 9 males. Samples of unknown sex were not used for this study.

4.3.3 Experimental design for temperature and CO_2 stress

The temperature and CO_2 conditions chosen for the manipulative experimental stress were based on previous studies with the hard coral *Acropora* specimen and which resulted in documented suppression of physiological parameters (Ogawa *et al.*, 2013) and apoptosis (Moya *et al.*, 2014). For the CO_2 treatment, natural seawater in a 500 L sump tank was acidified with 100% CO_2 using a pH computer (Aquamedic AT−Control, Germany) as described in Watson *et al.* (2012). To control for an effect due to the sump tank, a non−acidified 500 L sump tank was used. Following acidification, seawater was heated with in−line pond titanium heaters (Weinu, Taiwan) to 32˚C in two 60 L header tanks that were supplied with seawater from of the sump tanks by a pond pump (Bianco, Australia). Each of the 60 L header tanks fed seven 3 L aquaria in which the coral fragments were placed randomly, one coral fragment per tank (Appendix Figure 4.1). The flow−through seawater into each experimental aquarium was adjusted to 250 mL∫min to ensure maintenance of experimental conditions. Corals were kept at a 12∫12 h day∫night cycle with Sanyo lights (Sydney, Australia) connected to a timer clock.

After transferring the coral fragments into the experimental aquaria, temperature was increased in the respective 60 L header tanks from 27 ˚C to 32 ˚C over 48 h, pH was decreased over 6 h after the temperature had reached 32 ˚C. Temperature and pH_{NBS} (model MW102, Milwaukee, USA) were measured at least 3 times a day and coral performance monitored. The experiment time started when stressors had reached their experimental level, of a pH of 7.9 and temperature 32 ˚C, respectively. Samples for total DNA extraction and caspase activity assays were taken 1 and 12 days after the start, snap frozen in liquid nitrogen and stored at −80 ˚C until processing.

4.3.4 Total alkalinity and CO_2 partial pressure

Seawater chemistry analyses were performed using water samples taken daily from the four 60 L header tanks and preserved with mercury chloride, 0.04 % final concentration, 100 mL sea water with 40 µL saturated $MgCl_2$. Seawater chemistry was analysed following Miller *et al.* (2012). Briefly total alkalinity was determined by Gran titration with certified reference material (Batch 136, Dr A. G. Dickson, Scripps Institution of Oceanography). Total alkalinity of header tanks was used to calculate the pCO_2 in experimental tanks based on the

pH and temperature measurements taken for individual tanks during the midday measurements in CO2SYS (Pierrot *et al.*, 2006) using the NBS scale as pH scale and all other settings as standard (Table 4.1).

Table 4.1 Mean values (± SD) for temperature, pH, total alkalinity and pCO_2 for each experimental treatment over the course of the experiment.

Treatment	Temperature [˚C]	pH_{NBS}	Total Alkalinity [µmol/kgSW]	pCO_2 [µatm]
Control	27.6 ± 0.24	8.1 ± 0.02	2319 ± 10.2	504.9 ± 42.74
Temperature	30.9 ± 0.15	8.1 ± 0.02	2323 ± 4.5	544.7 ± 32.90
CO_2	27.5 ± 0.28	8.0 ± 0.01	2323 ± 13.8	776.8 ± 23.58
Temperature + CO_2	31.1 ± 0.17	7.9 ± 0.02	2325 ± 7.3	867.7 ± 39.39

Total alkalinity was measured in the four header tanks for each treatment condition. Temperature and pH were measured daily in experimental tanks of each experimental treatment. These daily measured parameters were used to estimate pCO_2 in CO2SYS.

4.3.5 Total DNA extraction and microbiome sequencing

Prior to DNA extractions, frozen tissues samples of both experiments were ground to powder in liquid nitrogen. Approximately 25 mg of ground tissue were used for total DNA extraction with the DNase Blood and Tissue kit (Qiagen, Australia) following the supplier's instruction. To avoid RNA contamination, an additional RNA digest was performed according to the manual's suggestions. DNA concentration was measured using the Qubit® HS DNA assay (Invitrogenƒ Life Technologies, Australia) and integrity verified on agarose gel.

To identify the microbial diversity, the V3 and V4 region of the prokaryotic 16S ribosomal RNA gene was amplified following the 16S library preparation guide for the lllumina MiSeq system. Briefly, the V3 and V4 regions were amplified with PCR primers including the lllumina overhang adapter sequences, which resulted in a PCR product of about 500 bp. DNA samples were diluted to 5 ngƒµL prior to PCR amplification. For each sample the PCR reaction was performed in triplicates. After purification, the concentration of each PCR product was determined (Qubit® HS DNA assay, lnvitrogenƒ Life Technologies, Australia) and triplicates for each sample were combined at an equimolar concentration of 2 nM. lllumina adapter sequences and dual–index barcodes were added to each PCR mix with the Nextera XT lndex kit (lllumina, USA) in a second PCR reaction. The prepared libraries were pooled at an equimolar ratio of 2nM after purification. Libraries were sequenced on MiSeq at James Cook University

using paired 300−bp reads and MiSeq v3 reagents (lllumina, USA). As an internal sequencing control, the library pool was spiked with 10 % PhiX control (lllumina, USA).

The sequencing run was monitored over BaseSpace and data were directly uploaded from the MiSeq machine. Sequencing resulted in approximately 1.86 million reads passing the filter with on average 38,000 reads per sample, ranging from 16,000 – 75,000 reads.

4.3.6 Microbiome analysis

Sequence data were available as demultiplexed reads on BaseSpace. The first 16 bases were trimmed of the forward reads and the first 18 of the reverse reads to remove the primer sequences. Further downstream analysis was conducted in QllME (Version 1.9) (Caporaso et al., 2010) and built−in functions. Prior to de novo OTU picking using mothur with 97 % sequence similarity (Schloss *et al.*, 2009; Schloss *et al.*, 2011), chimeric sequences were removed using USEARCH61 (Edgar, 2010). Cleaned sequences were aligned against the SlLVA database (SlLVA SSU Release 119, July 2014) (Quast *et al.*, 2013). Sequence data from sex−specific and stress experiments were analysed separately. The sex−specific microbiome sequencing resulted in 121369 OTUs with 2,263,516 counts. For the stress experiment a total of 34,947 OTUs were observed with 1,988,523 counts. However the counts varied from 81,204 to 218,374 counts per sample in the sex experiment and from 3,248 to 119,032 in the stress experiment. For statistical analysis, only OTUs with more than 15 counts in total were considered, which reduced the number observed OTUs to 2,578 and 3,509, respectively.

ln order to get an insight in the most abundant ribotyptes associated with *L. pauciflorum*, sequences of the 20 most abundant OTUs were extracted from the each data set and blasted (blastn) (Altschul *et al.*, 1997) against the NCBl database. To further specify the microbial composition, the core microbiome was analysed using the *compute_microbiome* function in QllME as suggested by Ainsworth *et al.* (2015). Based on previous studies (Philippot *et al.*, 2013) a representation of 51 % was chosen for the core microbiome analyses of the sex and the stress experiment. This implies if an OTU was not present in at least 51 % of the samples than it was considered as individual variability of colonies.

Sequencing reads were normalised using the relative log expression method proposed by Anders and Huber (2010) in edgeR (Robinson *et al.*, 2010). Following normalisation, counts were standardised by species and site and a multivariate correspondence analysis was performed with vegan (Oksanen, 2013, 2015). A multivariate correspondence analysis was chosen instead of a principle component analysis, as the data did not meet the requirements

for neither normality nor homogeneity of variance. The 20 most abundant OTUs were extracted from normalised read counts of the respective data set and used to generate a heat map with gplots (Warnes *et al.*, 2009; Warnes, 2015) and RColorBrewer (Neuwirth, 2011). Box and CA plots were produced using ggplot2 (Wickham, 2009). To generate the Venn diagrams the R package limma (Ritchie *et al.*, 2015) was used.

4.3.7 Apoptotic cell death

To investigate the host response to simulated environmental stressors increased temperature, low pH and the combination of increased temperature and low pH, the occurrence of apoptotic cell death was studied. The enzyme activity was measured using a Caspase−Glo 3ƒ7 assay Kit (Promega, Australia). To prepare sample extracts from coral tissue, ~ 15 mg frozen tissue powder were homogenized in 1.5 mL (1:100 (wƒv)) hypotonic extraction buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA and protease inhibitors (complete protease inhibitor cocktail, Roche, Australia; 1 pellet in 10 mL buffer)) using a FastPrep®−24 (MP Biomedicals, Australia) at program 3 twice with a 5 min interval on ice until the tissue was completely homogenized. Subsequently samples were centrifuged (5810−R, Eppendorf) for 15 min and 13,000 xg at 4 ˚C. 50 µL of supernatant and 50 µL reagent were mixed in a white−walled 96−microwell plate (NUNC F96) and incubated at 25 ˚C for 1 h. The light intensity emitted by each sample was measured in a GENios Pro Microplate Reader by Tecan (Männerdorf, Switzerland) in the luminescence mode. In each plate a sample blank containing 100 µL sample and reagent blank containing 50 µL buffer and 50 µL reagent were measured. To control reagent quality on subsequent days and comparability between different kits, one reference sample was prepared as 0.5 mL aliquots and deep frozen at −80 ˚C. One of these aliquots was measured once a day.

The kit contained a luminogenic substrate, including the tetrapeptide sequence DEVD, and a reagent optimized for caspase activity, luciferase activity and cell lysis. The DEVD sequence corresponded to a sequence in the poly−ADPribose polymerase, cleaved by caspase 3 and 7. Upon addition to a sample, cells were lysed and existing caspases cleaved of the substrate. The resulting “glow−type” luminescent signal was proportional to the amount of caspase activity present in the sample. Caspase activity was measured in relative light units (RLU) and normalized to protein concentration. Caspase activity was calculated as RLUƒ mg protein. Protein concentration was determined using the Qubit® Protein assay (Invitrogenƒ Life Technologies, Australia).

4.4 RESULTS

4.4.1 The microbial community associated with *Lobophytum pauciflorum*

A total of 2578 OTUs were identified to be associated with *Lobophytum pauciflorum,* derived from 2,161,800 sequencing reads with stringent quality checking and considering only OTUs with 15 or more reads. This microbiome profiling was derived from samples constituting both the exterior surface and interior tissues and was dominated by the class of Spirochaetes and the family of *Spirochaetaceae* representing on average 40 % of the retrieved sequences across all samples (Figure 4.1). The second most abundant taxonomic class was the γ–Proteobacteria, which constituted approximately 26 % of the recovered sequences. Within the γ–Proteobacteria, 21 % of the sequences were related to the family of *Endozoicimonaceae*. α–Proteobacteria related sequences accounted for 8 % of recovered microbial 16S rRNA gene sequences across all samples though these fell within a diverse array of different family groups, with *Rickettsiaceae* being the most abundant family representing 4 % of the assigned OTUs. Interestingly despite stringent sequence quality checking and comparisons to the SILVA microbial database, a high percentage of retrieved sequences (~ 22 %) could not be assigned.

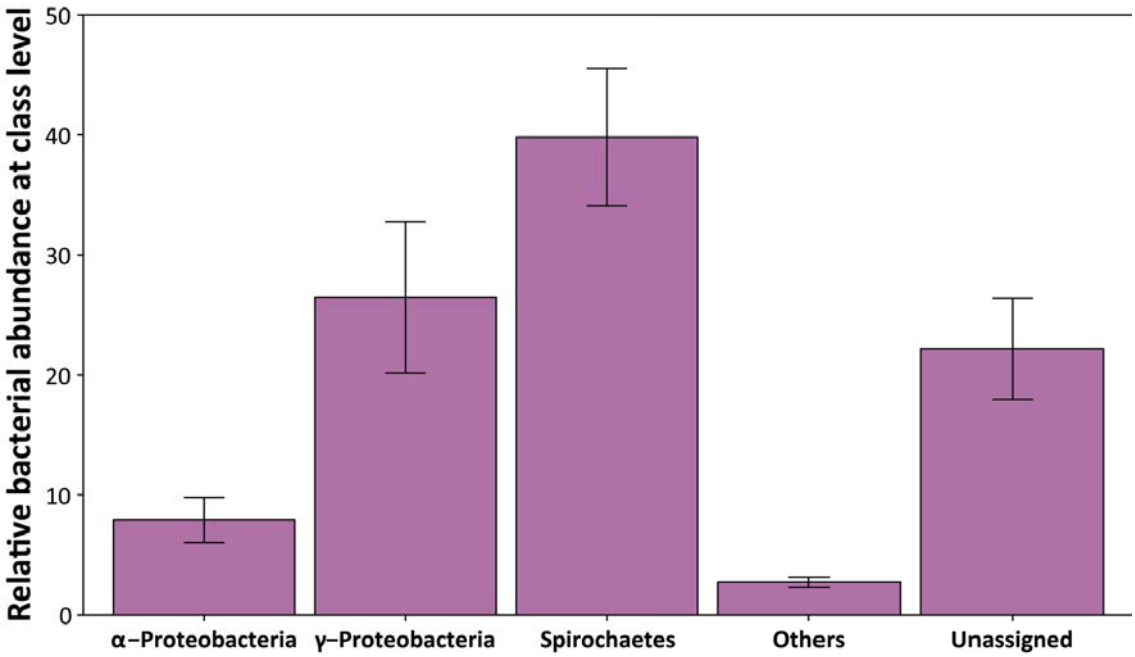


Figure 4.1 Relative abundance of the 2,578 OTUs contributing to the overall bacterial community structure of *L. pauciflorum* at class level. Mean relative abundance ± standard error, n = 17.

The core microbiome, which represented sequences retrieved from at least 51% of samples of *Lobophytum pauciflorum*, presented an almost identical microbial profile. A total of 832 OTUs were identified with sequences belonging to the Spirochaetes the most abundant, contributing 43 % of retrieved sequences and all sequences assigned to this class being members of the family *Spirochaetaceae* as well (Table 4.2). y–Proteobacteria represented on average 27 % of the bacterial core community across all samples with the *Endozoicimonaceae* being dominant (~23 % of assigned reads fell within this family). On average 7 % of the retrieved sequences were closely related to a–Proteobacteria with the majority of these sequences (4 %) belonging to the family *Rickettsiaceae*. Approximately 22 % of all sequences identifed as members of the the core microbiome were not assigned (Table 4.2). Since the bacterial diversity patterns within the core microbiome were highly similar to microbial patterns when all the data was investigated, futher analysis and interpretation is based only on this core microbiome.

4.4.2 Is there a sex-specific microbiome for *Lobophytum pauciflorum*?

A direct comparisons between male and female colonies demonstrated similar core microbiome profiles for the different soft coral sexes at the class taxonomic affiliation level (Table 4.2). The male microbial community was however characterised by a slightly higher relative abundance of Spirochaetes–related sequences though this was not significantly different to the female community profile (p = 0.14) (Table 4.2). At the family level, OTUs affiliated with the *Rhodobacteraceae* were about 4 times more abundant in male than in female corals, though this difference was not significant (p = 0.083). The female microbiome was characterised by an almost two times higher relative abundance of sequences that were unassigned, 29 % and 16 %, though this difference was not significant (p = 0.061) (Table 4.2). Further relative abundance of *Rickettsiaceae*–related sequences was also twice as high in female soft corals than in males, though the difference was not significant, due to high variation within each sex (p = 0.27).

Table 4.2 Mean relative abundance ± standard error of the 832 OTUs contributing to the core bacterial community structure of *L. pauciflorum* at family level, and distribution of relative abundances between female and male colonies, n = 8 female and 9 male soft coral colonies.

Class	Family	Relative % sequence abundance	Female	Male
a-Proteobacteria	*Rhodobacteraceae*	1.13 ± 0.41	0.45 ± 0.18	1.72 ± 0.71
	Rickettsiaceae	4.19 ± 2.04	5.86 ± 3.35	2.71 ±2.52
y-Proteobacteria	*Endozoicimonaceae*	22.70 ± 6.70	23.13 ± 10.01	22.31 ± 9.57
	Francisellaceae	1.50 ± 0.55	1.57 ± 0.68	1.43 ± 0.88
	Pseudoalteromonadaceae	0.68 ± 0.37	0.18 ± 0.10	1.08 ± 0.69
	Vibrionaceae	0.44 ± 0.20	0.25 ± 0.12	0.62 ± 0.37
Spirochaetes	*Spirochaetaceae*	42.51 ± 6.00	35.05 ± 8.81	49.15 ± 8.00
	Others	4.67 ± 1.01	4.16 ± 0.96	5.12 ± 1.75
	Unassigned	22.21 ± 4.20	29.34 ± 7.13	15.85 ± 4.05

The 20 most abundant ribotypes made up approximately 86 % of the total core microbiome based on their normalised counts (Figure 4.3). OTU 1 was closely related to an *Endozoicomonas* sp. sequence first identified in a marine sponge and represented 25% of all retrieved sequences (Figure 4.3). Of the 20 most abundant OTUs, 5 were closely related to *Endozoicomonas* spp. and accounted for 30 % of the core microbiome community (Figure 4.3).

OTU 2 was related to a *Spirochaetes* sp. sequence recovered from marine sediments and constituted 23% of the retrieved sequences from this study. Other OTUs 3, 4 and 5 were also closely related to Spirochaetes–affiliated sequences and together accounted for approximately 40 % of the community structure (Figure 4.3). Among the 20 most abundant OTUs were also 3 sequences, OTU 10, 14 and 17, that were closely associated with a *Cytophaga* sp. BHI80–3 sequence and accounted for 4 % of the relative abundance of the core microbiome (Figure 4.3). Although *Alteromonadaceae* were not represented among the most abundant families, OTU 7, which represented 2 % of the retrieved sequences, was closely related to an *Alteromonadales* sp. extracted from a marine sponge (Steinert *et al.*, 2014). *Ricksettiaceae* related sequences (OTU 8) were the most abundant family within the a–Proteobacteria, though only represented 2 % of retrieved sequences (Figure 4.3). Interestingly, OTU 2 was highly abundant within all *Lobophytum* samples, whereas the other OTUs showed colony specific variation in relative abundance (Figure 4.3).

A multivariate correspondence analysis of the core mirobiome structure with constraints for both sex and sampled colony explained 35.12 % of the variation. The three sampling locations (Cattle Bay, Little Pioneer Bay and Pelorus Island) significantly ($p < 0.05$) separated along the first and second constraint correspondence axis, which represented 16.67 % and 9.24 % of the variation, respectively, of the association between samples and microbial community (Figure 4.2). The male and female microbial communities were significantly different along the second constraint correspondance axis, $p < 0.05$ (Figure 4.2).

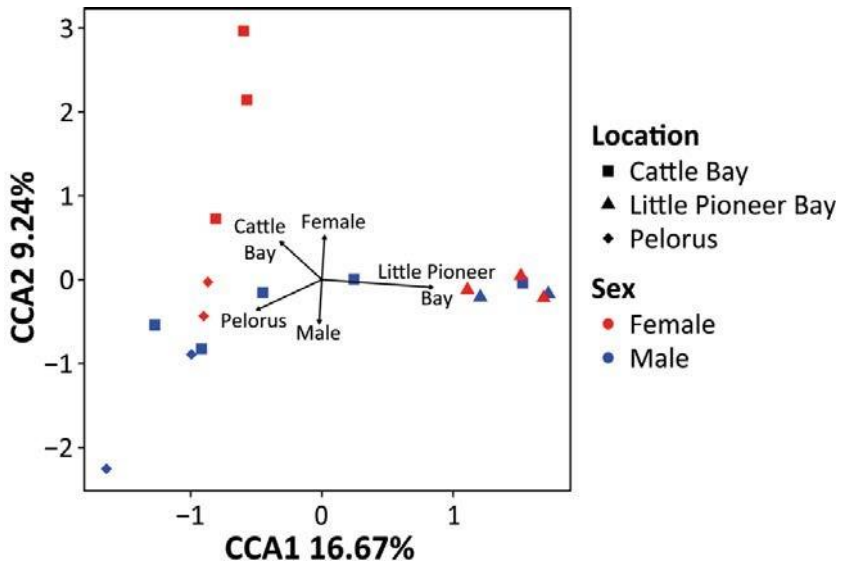
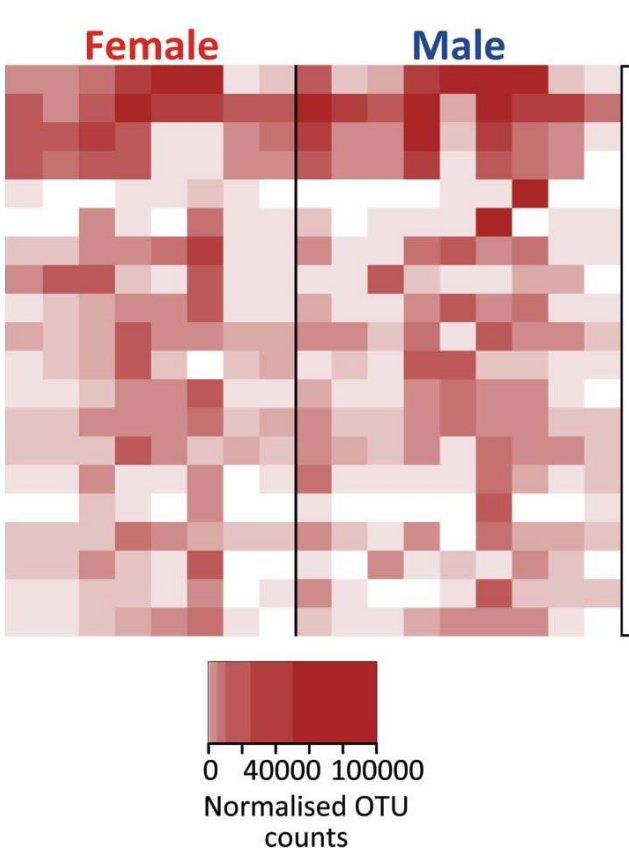


Figure 4.2 Correspondence analysis of all 832 OTUs comprising the core microbiome at 51 % representation. Percentages in the axis titles represent the percentage described by the axis. Squares represent colonies from Cattle Bay, triangles colonies from Little Pioneer Bay and diamonds for colonies from Pelorus. n = 8 for female samples, n = 9 for male samples.



	Relative abundance	86%	Nearest relative (Acc. Number -% similarity)	Class	Biological source	OTU length (bp)
OTU1	25%	*Endozoicomonas sp.* (JX442229 - 96%)	γ-Proteobacteria	Marine sponge (unpublished)	284
OTU2	23%	*Spirochaetales sp.* (AJ620502 - 91%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU3	7%	*Spirochaeta sp.* (AY337319 - 90%)	Spirochaetes	*Spirochaeta sp.* M6 (unpublished)	284
OTU4	5%	*Spirochaeta litoralis sp.* (NR_104732 - 86%)	Spirochaetes	*Spirochaeta litoralis* (unpublished)	279
OTU5	5%	*Spirochaetales sp.* (AJ620502 - 93%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU6	3%	*Spiroplasma sp.* (KF840734 - 89%)	Mollicutes	Sheep (unpublished)	284
OTU7	2%	*Alteromonadales sp.* (KF282361 - 95%)	γ-Proteobacteria	Marine sponge (Steinert *et al.*, 2014)	284
OTU8	2%	*Orientia tsutsugamushi* (AF479303 - 91%)	α-Proteobacteria	*Orientia tsutsugamushi* strain TC586 (unpublished)	284
OTU9	2%	*Endozoicomonas sp.* (JX442229 - 97%)	γ-Proteobacteria	Marine sponge (unpublished)	279
OTU10	2%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU11	1%	*Francisella hispaniensis* (KT281843 - 96%)	γ-Proteobacteria	Blood culture (unpublished)	284
OTU12	1%	*Endozoicomonas sp.* (JX442229 - 98%)	γ-Proteobacteria	Marine sponge (unpublished)	279
OTU13	1%	*Endozoicomonas montiporae* (NR_116609 - 97%)	γ-Proteobacteria	Scleractinean coral (Yang *et al.*, 2010)	284
OTU14	1%	*Cytophaga sp.* (AJ431238 - 94%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU15	1%	*γ-Proteobacteria* sp. (FN398075 - 98%)	γ-Proteobacteria	Giant archaea (Muller *et al.*, 2010)	284
OTU16	1%	*Spiroplasma sp.* (KJ470109 - 91%)	Mollicutes	River prawn (unpublished)	280
OTU17	1%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU18	1%	*α-Proteobacteria* sp. (DQ486492 - 89%)	α-Proteobacteria	Dinoflagellate (unpublished)	284
OTU19	1%	*Sagittula sp.* (KP297965 - 99 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpublished)	284
OTU20	1%	*Endozoicomonas sp.* (KJ573540 - 96%)	γ-Proteobacteria	Marine sponge (unpublished)	284

Figure 4.3 Taxa affiliation of the 20 most abundant OTUs of the core microbiome at 51 % representation. Colour intensity of the heat map represents the normalised counts for the each OTU in each sample for each sex. n = 8 females and 9 males.

4.4.3 Effects of environmental stress on the microbial community

The response of the microbial community associated with *Lobophytum pauciflorum* to environmental stress conditions including; (1) an increased temperature of 32 ˚C, (2) decreased pH of 7.9 and (3) a combination of increased temperature and decreased pH over a period of 12 days was investigated. Sequencing the 16S rRNA, allowed the identification of 3509 OTUs derived from 1,886,381 sequencing reads recovered from *Lobophytum pauciflorum* samples with stringent quality checking and considering only OTUs with 15 or more reads. The core microbiome at 51 % representation comprised 249 OTUs dominated by Spirochaetes related sequences, assigned to the family of *Spirochaetaceae*, which represented 34 % of the retrieved sequences (Table 4.3). The second most abundant class in the core microbiome were the y–Proteobacteria (22 % of retrieved sequences) dominated by *Alteromonadaceae*– and *Enterobacteriaceae*–related sequences (6 % and 5 % of the sequences, respectively) though *Endozoicomonaceae*–related sequences were also relatively abundant (4 % of retrieved sequences, Table 4.3). *Endozoicomonaceae*–related sequences increased by roughly 4 % in samples taken after 12 days of exposure to environmental stressors while abundance of *Alteromonadaceae*–related sequences decreased by about 4 % (Table 4.3). *Vibrionaceae* related sequences contributed to a minor extent (2 %) to the microbial profile of *L. pauciflorum*. The abundance of sequences assigned to this y–Proteobacteria family increased 3–fold between day 1 and day 12 of the experiment ($p < 0.05$), similar to the increase of *Endozoicomonaceae*–related sequences (Table 4.3). Approximately 15 % of all sequences were associated with a–Proteobacteria and the *Rhodobacteraceae*–related sequences (10 % of the retrieved sequences) were the most abundant at this family level. Only 5%, of the sequences were assigned to *Flavobacteriia*–related sequences, though the relative abundance was about 3 times lower after 12 days of exposure to environmental conditions than after 1 day ($p < 0.05$, Table 4.3). Approximately 15 % of the sequences could not be assigned to known 16S rRNA sequences in the database.

Correspondence analysis provided no evidence for the sampling time point or environmental stress conditions of temperature, pH or combined temperatture and pH influencing the *Lobophytum* microbiome (Figure 4.4 A). Further the culturing conditions did not alter the microbial community, relative microbial abundances at class level were similar in samples taken either 1 day or 12 days after the experiment start (Table 4.3). The clustering pattern within the microbial communities sampled was however explained by the colony that the microbiome profiles were originally sampled from (Figure 4.4 B). The explanatory power of

the correspondence analysis was limited with the first and second correspondence axis explaining only 13 % and 11 % percent of the variation, likely due to the high similarity of the microbial community between all samples. However small shifts in the minor groups of ribotypes associated with individual colonies appeared to be the principal driver in the seperation of samples of the correspondence analysis (Figure 4.4 B).

Table 4.3 Mean relative abundance ± standard error of the 249 OTUs contributing to the core bacterial community structure of *L. pauciflorum* at family level, and distribution of relative abundances between sampling time points. Light grey shading highlights significantly different relative abundances between period of exposure to experimental stress factors 1 day and 12 days, $p < 0.05$, n = 24 fragments for 1 day exposure and 23 fragments for 12 days exposure.

Class	Family	Relative % sequence abundance	1 days	12 days
Bacteroidia	*Marinilabiaceae*	2.35 ± 1.10	0.28 ± 0.15	4.51 ± 2.17
Flavobacteria	*Flavobacteriaceae*	4.92 ± 1.07	7.33 ± 1.78	2.41 ± 0.90
a-Proteobacteria	*Rhodobacteraceae*	9.96 ± 1.62	10.26 ± 2.11	9.66 ± 2.54
y-Proteobacteria	*Alteromonadaceae*	5.54 ± 1.02	7.20 ± 1.78	3.80 ± 0.85
	Endozoicimonaceae	4.15 ± 1.43	1.97 ± 0.75	6.42 ± 2.76
	Enterobacteriaceae	4.53 ± 1.71	5.82 ± 3.10	3.19 ± 1.40
	Vibrionaceae	1.89 ± 0.54	0.80 ± 0.36	3.03 ± 1.01
Spirochaetes	*Spirochaetaceae*	34.04 ± 5.22	31.83 ± 6.79	36.34 ± 8.10
Others		17.47 ± 2.90	17.06 ± 4.62	17.90 ± 3.49
Unassigned		15.15 ± 1.94	17.45 ± 2.52	12.75 ± 3.02

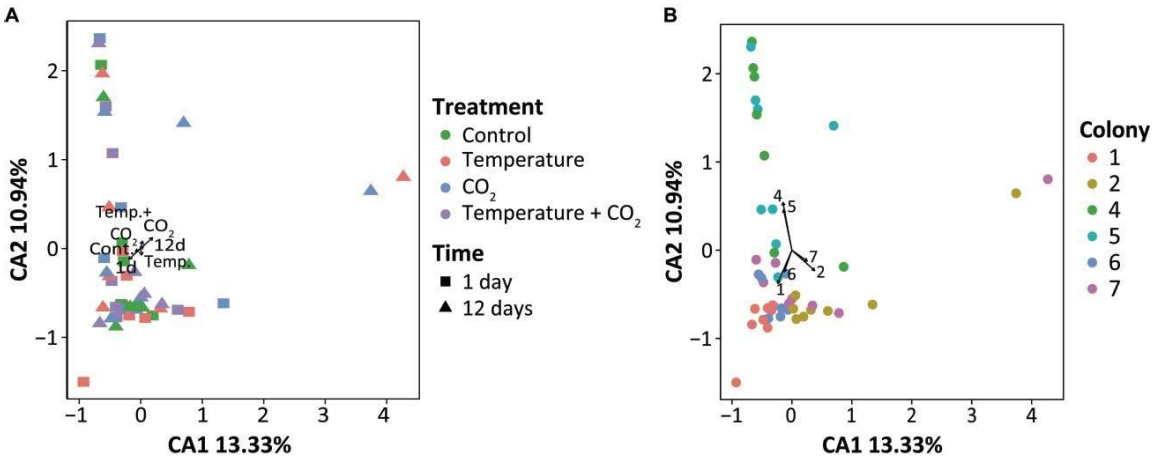
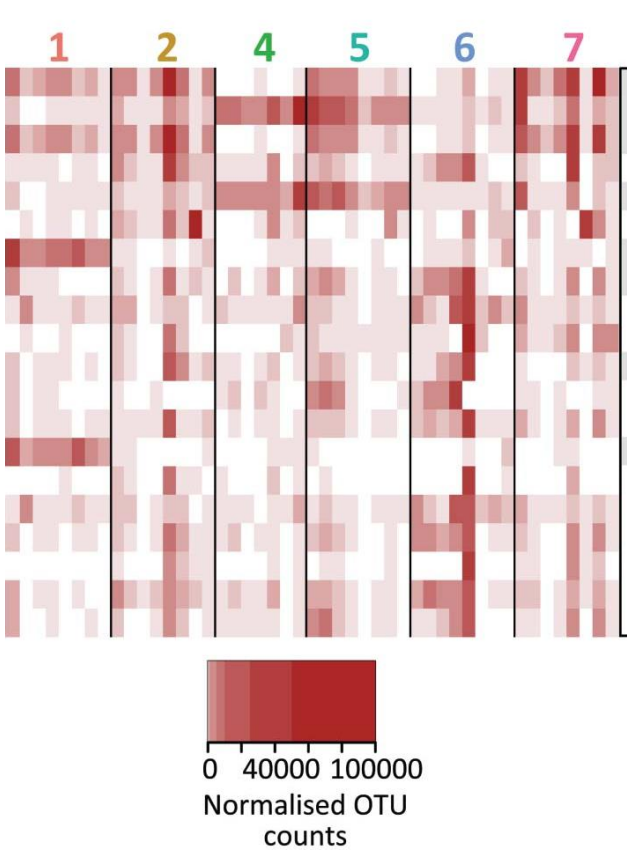


Figure 4.4 Correspondence analysis of all 249 picked and assigned OTUs of the core microbiome in *L. pauciflorum* fragments subjected to temperature and CO_2 stress. Percentages in the axis titles represent the percentage described by the axis. (A) Squares denote sampling after 1 day and triangles sampling after 12 days. Experimental stress conditions are differentiated by colours: Control in green, high temperature in red, high CO_2 in blue and combination of high temperature and high CO_2 in purple. n = 5 – 6 samples for each environmental stress per time point. (B) Each colony is described by its individual colour, n = 7 – 8 samples for each colony.

The 20 most abundant OTUs of the core microbiome accounted 48 % of the total retrieved sequence abundance (Figure 4.5). The three most abundant OTUs, OTU 1 – 3, with 6 – 7 % relative abundance each were associated with *Spirocheata* spp.–related sequences. Among the 20 most abundant OTUs, 4 OTUs (OTUs 1 – 3 and 7) were *Spirochaeta* sp.–related sequences made up 22 % of the microbial community profiles. Two ribotypes associated with a *Cytophaga* sp. BHI80–3 sequence contributed 4 % of the community. The relative abundance of y–Proteobacteria and a–Proteobacteria related sequences both represented approximately 6 % of retrieved sequences (Figure 4.5). y–Proteobacteria were represented by *Enterobacteraceae*– and *Alteromonadaceae*–related sequences, while a–Proteobacteria were only represented by *Rhodobacteraceae*–related sequences. Colony specific OTUs were also observed in this experiment, with OTU 7 and 14 being abundant in colony 1 and OTU 2 and 4 being more abundant in colonies 4 and 5. Of the 20 most abundant OTUs identified in this stress experiment 8 OTUs (OTUs 1, 2, 3, 5, 7, 8, 11, and 14) were also identified among the 20 most abundant OTUs in the sex experiment (described above) and assigned to the same sequences (Figure 4.3, Figure 4.5).



	Relative abundance	48%	Nearest relative (Acc. Number -% similarity)	Class	Biological source	OTU length (bp)
OTU1	7%	*Spirochaeta sp.* (AY337319 - 90%)	Spirochaetes	*Spirochaeta sp.* M6 (unpublished)	284
OTU2	7%	*Spirochaetales sp.* (AJ620502 - 91%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU3	6%	*Spirochaeta litoralis sp.* (NR_104732 - 86%)	Spirochaetes	*Spirochaeta litoralis* (unpublished)	279
OTU4	3%	Bacterium SCSIO13079 (KJ802185 - 100%)	Bacteria	Coral reef (unpublished)	284
OTU5	3%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU6	3%	*Marinifilum sp.* (KF650765 - 96%)	Bacteroidia	Marine Sediment (unpublished)	283
OTU7	2%	*Spirochaetales sp.* (AJ620502 - 93%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU8	2%	*Sagittula sp.* (KP297965 -99 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpblished)	284
OTU9	2%	*Enterobacter sp.* (KR779514 - 100%)	γ-Proteobacteria	Rabbitfish (unpublished)	284
OTU10	2%	*Lysinibacillus parviboronicapiens* (NR_041589 - 86%)	Bacilli	Soil from experimental field (Miwa *et al.*, 2009)	284
OTU11	2%	*Sagittula sp.* (KP297965 -98 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpblished)	284
OTU12	1%	*Flavobacterium sp.* (FM162878 - 96%)	Flavobacteriia	Coastal bacterioplankton (unpublished)	280
OTU13	1%	*Alteromonas macleodii* (KP074899 - 99%)	γ-Proteobacteria	Seawater (unpublished)	284
OTU14	1%	*Cytophaga sp.* (AJ431238 - 92%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU15	1%	*Lyngbya cf. confervoides* (AY599507 - 96%)	Oscillatoriophycideae	Lyngbya cf. confervoides VP0401	284
OTU16	1%	*Klebsiella pneumoniae* (CP006659 - 100%)	γ-Proteobacteria	Klebsiella pneumoniae ATCC BAA-2146 (Hudson *et al.*, 2014)	279
OTU17	1%	*Rhodobacteraceae sp.* (KM279016 - 99%)	α-Proteobacteria	*Coccolithus braarudii* (unpublished)	284
OTU18	1%	*Verrucomicrobia sp.* (CP010904 - 85%)	Verrucomicrobia	Hypersaline microbial mat (unpublished)	284
OTU19	1%	*Rhodobacteraceae sp.* (EF629844 - 100%)	α-Proteobacteria	Flowthrough aquaculture system (Mohamed *et al.*, 2008)	284
OTU20	1%	*γ-Proteobacterium sp.* (FJ952816 - 93%)	γ-Proteobacteria	Scleractinean coral (Rypien *et al.*, 2010)	284

Figure 4.5 OTU taxa affiliation of the 20 most abundant OTUs of 249 OTUs of the core microbiome for both sampling time points 1 day and 12 days after experimental stress conditions were established. Colour intensity of the heat map represents the normalised counts per sample for the respective OTU. n = 7 – 8 samples for each colony.

4.4.4 Apoptotic cell death

Caspase activity, as marker for host response to stress and as part of the apoptotic immune response system, was measured in the soft coral fragments subjected to temperature and CO_2 stress, though did not vary between sampling time points, 1 d vs 12 d, or between environmental stress conditions (Figure 4.6 A). Within treatment and time point variation was higher than between treatments and time points (Figure 4.6 A). In addition, the variability between coloniesƒgenotypes was greater than the impact of the environmental stress, making the stress response of the host seem to be rather driven by each colony than by the stresses applied (Figure 4.6 B). A linear model could not explain these differences, potentially due to missing information about other physiological factors or sex.

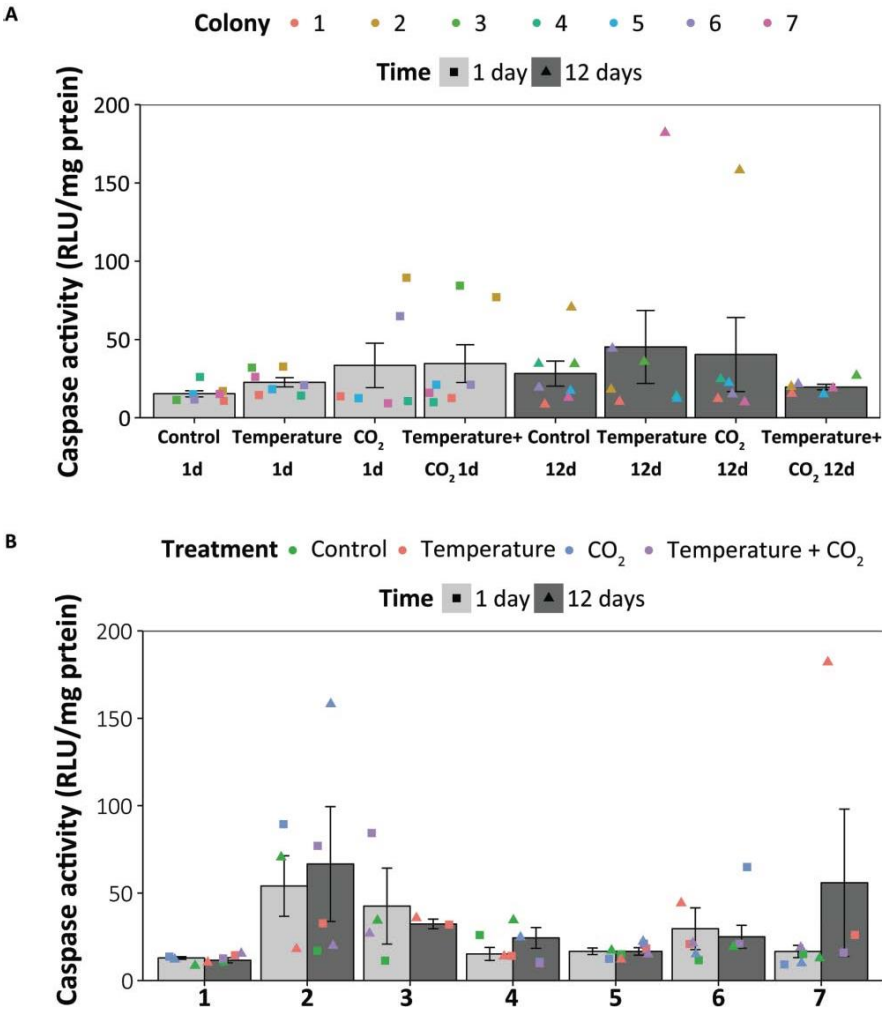


Figure 4.6 Mean caspase activity in relative light units (RLU) per mg protein for (A) each experimental condition and sampling time and for (B) each colony of origin with standard error in *L. pauciflorum*. Light grey bars represent samples taken after 1 day and dark grey bars samples taken after 12 days under experimental conditions. Colours stand for the colony of origin (A) or the different environmental conditions (B). Squares and triangles in (B) denote the sampling time point of 1 day and 12 days, respectively. n = 6 – 8 samples for the combination of experimental condition and time point and colony of origin.

4.5 DISCUSSION

4.5.1 The microbial community associated with *Lobophytum pauciflorum*

The microbial communities of soft corals are poorly understood though, like other marine invertebrates, likely important within a functioning healthy colony (Rohwer and Kelley, 2004; Bourne *et al.*, 2008). This study established a baseline understanding of the microbiota associated with *Lobophytum pauciflorum* and demonstrated the high relative abundance of Spirochaetes, which represent approximately 40 % of the retrieved sequences. Abundant Spirochaete–related sequences have been found in other coral microbiome studies such as the red coral *Corallium rubrum* from the Mediterranean Sea (van de Water *et al.*, 2016) and scleractinian corals transplanted in close proximity to a high effluent fish culture in the Philippines (Garren *et al.*, 2009). Low abundances of Spirochaetes were also detected on a site specific dependant pattern in *Stylophora pistillata* colonies at the GBR (Kvennefors *et al.*, 2010) and in the gorgonian coral *Plumarella superba* from the Aleutian islands (Gray *et al.*, 2011). Further Spirochaetes were identified as a small constituent of the endosymbiotic core microbiome in three mesophotic hard corals (Ainsworth *et al.*, 2015). In terrestrial insects, Spirochaetes are commonly observed in the hindgut of termites establishing an obligate symbiosis that has coevolved (To *et al.*, 1978; Berlanga *et al.*, 2007). The localisation of this dominant bacterial group associated with *L. pauciflorum* tissues is unknown, though as observed with other invertebrates, it may be within the tissue layers rather than as epibionts on the outside of tissues. *Lobophytum* sp. have also been shown to exhibit high antimicrobial properties due to secondary metabolites produced by the coral host (Yan *et al.*, 2010a; Yan *et al.*, 2010b; Yan *et al.*, 2011), which may further indicate an internal rather than external pattern of colonisation of the Spirochaetes. Spirochaetes have also been suggested to have an important role in structuring the microbial community of *C. rubrum* by secretion of antibiotics (van de Water *et al.*, 2016). However, further studies including fluorescent *in situ* hybridisation targeting this specific group with oligonucleotides probes is warranted to confirm their localisation and further inform on any functional role.

y–Proteobacteria were the second most abundant class of sequences associated with *L. pauciflorum* (~ 26% relative abundance, Figure 4.1, Table 4.2) and the majority of sequences within this class were affiliated with the *Endozoicimonaceae* (~ 23 % relative abundance; Table 4.2). *Endozoicomonaceae* are a family within the y–Proteobacteria that is commonly found in association with hard and soft corals but also other marine invertebrates (Bayer *et al.*, 2013b; Bourne *et al.*, 2013; Nishijima *et al.*, 2013; Lema *et al.*, 2014a; La Rivière *et al.*, 2015). Previous

genome sequencing of symbiotic *Endozoicomonas* strains suggested a functional role in nitrate cycling and a symbiotic relationship with the host (Neave *et al.*, 2014). An intimate relationship of host endoderm cells and aggregations of *Endozoicomoas* were identified for the Red Sea corals *Stylophora pistillata* and *Pocillopora verrucosa* also suggesting an endosymbiotic relationship (Bayer *et al.*, 2013b; Neave *et al.*, 2016). Further recent studies suggest a close coevolution of *Endozoicomonas* strains and gorgonian species (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015) and therefore similar symbiotic relationships with this bacterial family and *L. pauciflorum* may exist. However specific *in–situ* localisation studies combined with host–microbe functional analyses are required to confirm these relationships. Interestingly, localisation of microbial communities associated with scleractinian corals is also currently poorly understood but are an essential step in interpreting a central roles for of these bacterial groups within the holobiont.

Among the a–Proteobacteria, *Rickettsiaceae*–related sequences were identified as the most abundant (4 % relative abundance, Table 4.2). In scleractinian corals, cell–associated microbial aggregates were found to be *Rickettsia*–related and were suggested to play an important role as facultative secondary symbionts (Work and Aeby, 2014). In other invertebrates such as the whitefly, *Rickettsia* sp. were found in gut and follicle cells, suggesting an endosymbiotic relationship between fly and bacteria (Gottlieb *et al.*, 2006). *Rickettsia* were identified to be transmitted vertically but also horizontally within the population, suggesting that infection leads to an advantageous phenotype in the whitefly though the exact function that these bacteria have in the biology of the whitefly remains to be determined (Gottlieb *et al.*, 2006). A contrasting example from the pea aphid revealed that *Rickettsia*–infection had negative effects on some components of the host fitness when compared to *Rickettsia*–eliminated strains from the same genetic background (Sakurai *et al.*, 2005). In *Lobophytum* and well as many other marine invertebrates, the role of *Rickettsiaceae*–related sequences needs to be further explored. It is likely that this bacterial family is also localised among cell–associated microbial aggregate as in scleractinian corals (Work and Aeby, 2014), however their functional role within the octocoral microbiome needs to be further investigated potentially through a metagenome sequencing approach.

Lobophytum colonies were further associated with low abundant *Pseudoalteromonadaceae* and *Vibrio*–related sequences, 0.7 % and 0.5 %, respectively (Table 4.2). Both *Pseudoalteromonas* sp. and *Vibrio* sp. associated sequences are widely recovered from scleractinian coral microbiome studies and *Vibrios* in particular often associated with compromised coral health (Bourne *et al.*, 2008; Vega Thurber *et al.*, 2009; Séré

et al., 2013). However *Vibrionaceae*–related sequences may also be a normal constituent of the coral microbiome and Ainsworth *et al.* (2015) suggested that this family might rather be associated with the surface mucus. Due to the antimicrobial activity of the secondary metabolites discovered in *Lobophytum* sp. (Zhao *et al.*, 2013) it is possible that members of the *Vibrionaceae* recovered among the sequences in *L. pauciflorum* are also associates with the soft coral mucus. In *Pocillopora meandrina* planulae *Pseudoalteromonas* strain HIMB1276 were found to be associated with cells of the outermost ectoderm possibly involved in settlement, which might indicate that *Pseudoalteromonas* sp. play a role in the induction of settlement and metamorphosis (Apprill *et al.*, 2012). Tetrabromopyrrole isolated from *Pseudoalteromonas* might induce settlement in *A. millepora* highlighting the potential importance for *Pseudomonadaceae* during settlement in scleractinian corals (Tebben *et al.*, 2011). Though the association might also have a beneficial role in adult corals due to the high abundance recovered in adult *A. millepora* (Littman *et al.*, 2009), the low abundance in *Lobophytum* recovered in this study suggests that these bacteria might only be epibionts associated with the soft coral mucus or particles.

4.5.2 Is there a sex-specific microbiome in *Lobophytum pauciflorum*?

Studies on *Lobophytum compactum* and *Sarcophyton glaucum* showed sex–specific differences in the UV protective mycosporine–like amino acids (Michalek–Wagner, 2001) and complimentary metabolite concentration (Fleury *et al.*, 2006) between female and male colonies. Sex–specific microRNAs have also been identified in *N. vectensis* (Moran *et al.*, 2014), and therefore sex–specific microRNAs might also exist in *L. pauciflorum,* differentially regulating the innate immune system leading to different microbial communities in female and male colonies. On the basis that physiological differences between sexes might be regulated by an altered immune system in bilateria (Klein, 2000; Peng *et al.*, 2005; Love *et al.*, 2008; Nunn *et al.*, 2009), the potential for sex–specific differences in the microbial community in *L. pauciflorum* was investigated in this study. At class and family level, the microbial profiles associated with female and male colonies were highly consistent and the 20 most abundant OTUs that were classified taxonomically also displayed no differences in relative abundance between sexes. Though we did not see any difference in the dominant communities of the microbiome between male and female soft corals, sampling at different times of the year, for example during gametogenesis periods, might provide a different picture. In the current study, samples were taken 1 month prior to spawning when gametogenesis was complete, and therefore temporal sampling would be beneficial.

Multivariate correspondence analysis at the OTU level however did reveal a significant difference between sampling sites and sexes of *Lobophytum* colonies ($p < 0.05$, Figure 4.2). The difference between sampling sites was mainly driven by a varying relative abundance of γ–Proteobacteria and Spirochaete–related sequences, however replication for sampling sites was not evenly distributed and therefore this patterns should be viewed cautiously (e.g. 8 fragments from Cattle Bay and 4 from Pelorus, Appendix Table 4.1). The sex specific difference might have been driven by OTUs that were not assigned to any known bacterial 16S rRNA sequence, with approximately 30% of sequneces in female colonies unassigned verses 16% in male colonies. Different microbial abundances between inshore and mid–shelf sampling sites were detected in *A. millepora* where microbial communities from inshore reef corals were dominated by γ–Proteobacteria–related sequences while in offshore reef coral microbiomes Bacilli–, α–Proteobacteria–related sequences were more abundant (Lema *et al.*, 2014b). The three sampling sites chosen for this experiment on *L. pauciflorum* can all be considered as inshore reefs. Considering the large individual variation within sampling sites and the low explanatory power of the correspondance analysis it is likely that the differences detected are rather driven by colony specific differences (Figure 4.3, Figure 4.2, Appendix Table 4.1).

There are few microbiome studies on octocorals and therefore these habitats provide the potential for the discovery of yet undescribed sequences from a novel environment. Sequencing errors may also be a factor in the inability to assign taxonomy, though OTUs were only considered if 15 or more reads were clustered and it is unlikely that errors would disproportionately affect sequences recovered from female colonies. Further explorations of the taxonomic origin of these sequences are required potentially through fluorescent *in situ* hybridisation (FISH) and metagenome sequencing approaches. This study focused on the microbial community of the holobiont and did not differentiate between different coral tissues. Secondary metabolites of *Lobophytum* sp. have been described to have antimicrobial properties (Zhao *et al.*, 2013) it is therefore possible that the detected differences between sampling sites are due to particles attached to the outside of the collected fragments rather than different internal tissue communites. Future studies should also investigate different tissue microhabitats, which might reveal interesting differences at colony or tissue level. The importance of rare OTUs was highlighted by Ainsworth and colleagues (2015), when analysing the core microbiomes of different scleractinan coral tissues where they found that bacterial phylotypes with a potentially important endosymbiotic function were of low abundance in the holobiont community (< 5%) but very abundant in the endosymbiotic community (>20 %) (Ainsworth *et al.*, 2015).

4.5.3 Effects of environmental stress on the microbial community and host response

Subjecting *L. pauciflorum* fragments to different environmental conditions including increased seawater temperature and lower pH did not shift the microbial community structure over an experimental period of 12 days. The microbial community was not altered by the environmental conditions and nor were the main microbial associates influenced by the length of the experiment (Table 4.3). Measurement of caspase activities, a marker for host response to stress as part of the apoptotic immune response system, also showed no significant changes under the experimental conditions over the course of the experiment. These results indicate that *L. pauciflorum* demonstrates high resilience to short term environmental stress events at both the host and microbiome levels. In contrast, Gorgonian species subjected to short term temperature stress (1 – 4 h) were found to increase expression of heat shock proteins suggesting an upregulation of protective mechanisms as a stress response and potential for development of tolerance (Wiens *et al.*, 2000; Kingsley *et al.*, 2003). Studies on stress responses of scleractinian corals have also shown sensitivity to CO_2 and temperature stress in adult colonies of *Porites compressa* where microbial community shifts from a healthy community to one that is often found in diseased corals (Vega Thurber *et al.*, 2009). Juvenile *A. millepora* subjected to elevated CO_2 stress for a 'prolonged', 9 d, period on the other hand showed the capacity to acclimate to elevated CO_2 conditions via suppression of apoptosis among others (Moya *et al.*, 2014). A high variability between alcyonacean species in susceptibility to bleaching was found during the mass bleaching event at the GBR in 1998 (Goulet *et al.*, 2008a). A moderate bleaching susceptibility for the family of Alcyoniidae, to which *L. pauciflorum* belongs (Goulet *et al.*, 2008a), together with a potentially similar physiological capacity to induce fast protein protection from denaturation or thermal deactivation under heat stress (Morimoto *et al.*, 1996; Nathan *et al.*, 1997) observed in gorgonians (Wiens *et al.*, 2000; Kingsley *et al.*, 2003), could explain the resilience of *L. pauciflorum* to the environmental stressors applied in this study. An unaltered apoptotic activity in *L. pauciflorum* might hence also reflect the capacity of soft corals to withstand CO_2 stress for a short period of time.

The microbial profiles of *L. pauciflorum* were consistent across two separately conducted experiments in this study, highlighting the dominant abundance of Spirochaete– and *Endozoicomonas*–related sequences and providing a baseline understanding of the microbial communities associated with *Lobophytum*. In addition to members of Spirochaetes and *Endozoicomonas*, members of 5 families with a relative abundance below

5 % were detected (Table 4.3) suggesting that the microbial biomass microbial community in *Lobophytum* is possibly low, similar to findings in *C. rubrum* where the core microbiome is composed of only 12 OTUs (van de Water *et al.*, 2016). The dominant relative abundance of *Endozoicomonas* and Spirochaetes might indicate that these are internal associates whereas members of the other families are possibly epibionts or associated with particles attached to the surface favouring high intercolonial variation. Together with data of species−specific octocoral−*Endozoicomonas* associations (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015) and the low diversity of the core microbiome of *C. rubrum* (van de Water et al., 2016) suggest that similar to species specific microbial communities in scleractinian corals (Rohwer *et al.*, 2002; Rohwer and Kelley, 2004), octocoral microbial profiles might also be highly species specific. However further tissue specific analysis of microbial communities will give more information on tissue localisation of microbial associates. High variability between genotypes and hence colony specific response to environmental parameters are a common observation when working with marine invertebrates. The sampling design for this experiment accounted for several replicates of each colony allowing the reduction of the inter−colonial variation. Replication at colony level further enabled the detection of a colony specific response of microbial communities and caspase activities to the environmental stressors rather than a stress specific response as the variation between the colonies was lower than within a colony (Figure 4.4, Figure 4.5, Figure 4.6). High individual variability has been previously observed in differential gene expression studies in *Acropora millepora*, limiting the detection of significant responses (Bay *et al.*, 2009; Seneca *et al.*, 2010; Bertucci *et al.*, 2015).

However the lower relative abundance of *Endozoicomonaceae*−related sequences in the stress experiment is potentially due to culturing of sampled fragments in a recirculating system for 2 months prior to conducting the experiment. However OTUs affiliated with *Endozoicomonaceae* were common and still represented 4 % in the microbiome of the aquarium manipulated corals. Transferring sponges from the wild into an aquaculture system induced a significant shift in microbial communities between wild and cultured sponges (Mohamed *et al.*, 2008). Notably the abundance of a− and y−Proteobacteria was reduced in sponges kept in culture for up to 2 years though the shift occurred after 6 months of culturing. Returning the coral fragments to an open system of ocean water appeared to have reversed this effect as *Endozoicomonaceae*−related sequences increased over the course of the experiment, for a full 'return' of the initial microbiome the experiment was probably not conducted long enough. Shifts in microbial communities as response to changing

environmental conditions were also observed in scleractinian corals (e.g. Bourne *et al.*, 2008; Vega Thurber *et al.*, 2009) and similar patterns are likely in *L. pauciflorum.*

4.6 CONCLUSIONS

This study provided a baseline understanding of the microbial community associated with the gonochoric soft coral *Lobophytum pauciflorum* and its susceptibility to environmental stress. The microbial community was dominated by Spirochaete–related sequences. A Spirochaete–dominated microbial community is a novelty for anthozoan species, however consistent for *L. pauciflorum* as it was recovered across two individually conducted experiments. All of the Spirochaete related sequences were assigned to the same genus of Spirochaete. The second most abundant bacterial class were y–Proteobacteria with the majority being assigned to *Endozoicomonaceae* at family level, which are commonly associated with marine invertebrates (i.e., Bayer *et al.*, 2013a; Nishijima *et al.*, 2013; Neave *et al.*, 2014). Sex–specific differences were not observed at class and family level however multivariate analysis at OTU level suggested colony specific assemblages that might be linked to different sexes. Short term environmental stress did neither affect the apoptotic activity as measure of the immune response, nor the composition of the microbial community in the *L. pauciflorum*. Analysing the caspase activity and microbial community showed that apoptotic response and microbial composition of the associated communities were colony specific. This suggests that the microbial community of *Lobophytum* is resilient to short term environmental stresses.

In situ hybridisation techniques are a powerful approach to determine the localisation of bacterial strains within the tissue. Future studies on *Lobophytum* using these techniques will allow the localisation of Spirochaete– and *Endozoicomonas*–related sequences within the soft coral tissue and help identification as ecto– or endosymbiont. Successful localisation of associated microbiota within *L. pauciflorum* will enable comparison to localisation in scleractinian corals. Culturing experiments followed by genome sequencing might give first conclusions on the potential functional role of these bacteria within the holobiont. To clarify this function, metagenome sequencing will enable the detection of possible metabolic functions looking at gene predictions together with additional information on taxonomic affiliations. This study identifies a new important microbial associate in soft coral biology by unravelling the Spirochaete–dominated microbial community and offers insight in a microbial community resilient to short term environmental stressors.

Chapter 5 General Discussion and Conclusions

5.1 From coral spawning to microbial communities – a tentative approach for comprehensive coral molecular biology

5.1.1 Reproductive strategies

Following the spawning behaviour of *Fungia concinna* over 4 years allowed the observation of bidirectional sex change in this species, which is only the second time that this has been observed in fungiid corals. In contrast to the accompanied observation of a size−related sexual dimorphism in *Ctenactis echinata* (Loya and Sakai, 2008), such a pattern was not noticed in this study on *F. concinna*. In addition, studies on *F. scutaria* and *F. granulosa* also identified these as protandrous, size related sexual dimorphism occurring in the former species but not in the latter (Kramarsky−Winter and Loya, 1998; Loya and Sakai, 2008). These combined observations highlight the sexual plasticity occurring within this coral family, but are consistent with the general pattern of smaller individuals being male. Studying spawning behaviour together with monthly tissue sampling allowed determining the onset of oogenesis in February and limiting the time window for a possible sex change to 2 − 3 months, starting shortly after the spawning event. To determine the exact time point of the initiation of sex change, more long term monitoring of a larger number of individuals and more frequent tissue sampling are required. This, however, is challenging with solitary corals such as *F. concinna* as the effects of repeated, e.g. monthly, tissue sampling have not been investigated, yet. To allow repetitive sampling of the same specimen for molecular purposes, it is necessary to collect small fragments using a hammer and chisel, a complication being that these corals are prone to shatter upon harsh physical contact. The repair of tissue damage resulting from frequent sampling might impact on sex change processes, by diverting energy resources that might otherwise be available for reproduction. Kramarsky−Winter and Loya showed however that *F. granulosa* if injured at the onset of gametogenesis, continued gametogenesis (Kramarsky−Winter and Loya, 2000). In their study, tissue lesions were induced using an air pick keeping, the skeleton intact, so using potentially more stressful sampling methods could have a stronger impacts on reproductive output and spawning behaviour.

The suggestion that *Lobophytum pauciflorum* might spawn earlier than *F. concinna* and *Acropora millepora* is based on the observation of different spawning intensities between October and November 2012 and missing spawning activity in November 2013. Further mature

gametes were observed in *Lobophytum* as early as late September from 2013 − 2015. In order to make more general conclusions, the spawning behaviour and gametogenesis of *L. pauciflorum* at different locations along the GBR should be monitored over multiple gametogenic cycles and variable environmental conditions also considered. Severe bleaching, for example, was shown to reduce reproductive output of the congeneric soft coral *L. compactum* for at least 20 months (Michalek−Wagner and Willis, 2001a). Further variation in spawning activity andfor intensity could also be responsible for the observation of unspawned oocytes in January 2014 after a potentially less active spawning in 2013. Whereas the colonies studied might have been more active during the spawning event in 2014, hence no unspawned material was observed afterwards. Variations in fecundity and recruitment were observed in three *Acropora* species on both spatial and temporal scales along 5 sectors of the GBR (Hughes *et al.*, 2000). Taking the reduced reproductive output of *L. compactum* in moderately and severely bleached colonies and the observed variations in fecundity in *Acropora spp.* into consideration, it is very likely that the differences in spawning activity and numbers of remaining oocytes in *L. pauciflorum* were due to environmental differences between the years. When working on spawning andfor early development in *L. pauciflorum*, gamete development and environmental conditions should be closely monitored as spawning time and reproductive output might depend on these.

5.1.2 Early development

Investigating early development in *L. pauciflorum* showed that unsynchronised and various morphological shapes and cleavage patterns during the first 24 h of development were similar to those observed in previously studied Alcyonacea (Matthews, 1917; Benayahu, 1989; Dahan and Benayahu, 1998). The high yolk content generally found in Octocorallia might indicate the tendency for superficial cleavage (Mergner, 1971) favouring the precedence of nuclear divisions before cytosolic divisions in *L. pauciflorum,* leading to the initial formation of a syncytium followed by the appearance of an embryo with more than 2 blastomeres. This hypothesis could be tested using nuclear staining methods during the early developmental phase. Additionally, the mode of gastrulation in *L. pauciflorum* remains to be determined. Gastrulation stages similar to the 'fat donut' described for *A. millepora* and *N. vectensis* (Babcock and Heyward, 1986; Ball *et al.*, 2002a; Fritzenwanker *et al.*, 2004; Kraus and Technau, 2006) were not observed in this study or previous studies on soft corals. However, if, as has been suggested (Wilson, 1883; Matthews, 1917), gastrulation in Octocorallia occurs via delamination rather than by invagination (as in Hexacorallia; Babcock and Heyward, 1986; Kraus and Technau, 2006), this might be a much less obvious process.

Using next generation sequencing technology, transcriptome data were generated for 11 different life history stages, from unfertilised eggs to adults, of *L. pauciflorum* and used to investigate the molecular bases of soft coral development. There have been only two previous transcriptomic studies of octocorals (Burge *et al.*, 2013; Pratlong *et al.*, 2015), in both cases based on adult specimens of *Gorgonia ventalina* and *Corallium rubrum,* respectively. Even though both published studies have better assembly characteristics and annotation statistics than those generated for *L. pauciflorum* in this study, the availability of developmental data is a major advantage in the present case. Further, developmental comparisons allowed the identification of soft coral homologs of genes implicated in calcification in other organisms (e.g. Lowe *et al.,* 1990; Hewett−Emmett and Tashian, 1996; Fukuda *et al.,* 2003; Ramos−Silva *et al.,* 2013) that were upregulated during settlement/metamorphosis. *In situ* hybridisation data for these genes should be a priority for future work, as it could provide new insights into the evolution of calcification processes in the Cnidaria.

5.1.3 Microbial community

Recognising the importance of microbial communities for the wellbeing or illness of the host organisms, from Cnidaria to vertebrates, and establishing the idea of the metaorganism or holobiont, combining the host and the associated prokaryotes, eukaryotes and viruses and their interactions (Bosch and McFall–Ngai, 2011; Bosch and Miller, 2016b), has changed the way microbes are perceived. Studies of microbial communities associated with soft corals are limited, however, investigations on gorgonians have established a close and possibly symbiotic relationship with members of the y–Proteobacteria genus *Endozoicomonas* (Sunagawa *et al.*, 2010; Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). *Endozoicomonacea* are also commonly associated with hard corals and many other marine invertebrates (Thomas *et al.*, 2009; Littman *et al.*, 2011). While *Endozoicomonacea*–related sequences were also found abundantly in *L. pauciflorum*, the microbiome of this soft coral was dominated by Spirochaete–related sequences. A Spirochaete–dominated microbial community has so far only been identified in the red coral *Corallium rubrum* in the Mediterranean Sea (van de Water *et al.*, 2016); Spirochaetes do occur in hard coral microbiomes but are typically only minor components of these. The novel nature of the Spirochaete–soft coral association requires further investigation; the location of the bacteria (within or outside the soft coral tissue) is of particular interest. Metagenomic data should shed light on potential functions of these bacteria. As Spirochaetes are typically of low abundance in hard corals (Ainsworth *et al.*, 2015), it is tempting to speculate that this association might be soft coral specific. In addition to transcriptome data, microbial studies on early life history stages of *A. millepora* showed that these stages were highly diverse and variable but stabilised later on (Lema *et al.*, 2014a). Findings in *Hydra* highlight a similar pattern, the adult microbiome is established within the first weeks of development (Franzenburg *et al.*, 2013) and stable throughout time (Fraune and Bosch, 2007). In addition to microbial studies on adults, it would be interesting to see if the microbial community of *L. pauciflorum* changes throughout development and when Spirochaetes start to dominate the community.

5.2 Establishing *Lobophytum pauciflorum* as new model for soft coral biology – Nightmare or challenge?

After studying the reproductive cycle and detecting the most efficient technique for gamete collection (see Figure Appendix 3.7), it was possible to collect enough material to also study the molecular characteristics of development in *L. pauciflorum*. Challenges in developing methods for gamete collection and missing spawning events during the initial period of this study restricted the extent to which it was possible to conduct molecular analysis of soft coral development. Though this thesis provides a general overview of this process, it was not possible to perform *in situ* hybridisation studies due to lack of time. The development of a suitable protocol for localisation of expression of key genes involved in soft coral development is in progress. On the other hand, this thesis provides the basis for future investigations. Before *L. pauciflorum* can, however, be considered as new model for soft coral development, it is important to answer vital questions on time point and mode of first cell division but also to understand the process of gastrulation and when it occurs. Preliminary attempts to determine the number of nuclei present in fertilised eggs by 4',6−Diamidin−2−phenylindol (DAPI) staining proved unsuccessful, most likely due to the high yolk content and low permeability of the eggs. For future studies, focussing on first cell cleavage and gastrulation, sufficient material would need to be collected for morphological and immuno−histochemical analyses. For example, sampling at 30 min time intervals starting with fertilisation and stopping after 75 % of the oocytes started dividing for the first part. For the gastrulation study, embryos should be sampled hourly from the time point when individual blastomeres can no longer be identified anymore through the various shape changes with irregular surface, until the surface becomes smooth again. Histological sectioning will hopefully provide insights into the mode of gastrulation and allow its timing to be more accurately determined. Closer investigations of the settlement process might enable identification of settlement cues such as the neuropeptide of the LW−amide, which could help to synchronise this process and facilitate comparison of underlying mechanisms with other cnidarians. Understanding the initiation of the calcification process in settled *Lobophytum* has the potential to allow comparisons with Scleractinia, which deposit calcium carbonate as aragonite rather than as calcite. Comparisons with calcisponges, which also deposit calcite crystals (Clarke and Wheeler, 1922), could also provide highly informative.

In addition to the developmental dimensions of this thesis, studies of the microbial community associated with *L. pauciflorum* presented here raise a number of new questions.

Future studies should investigate the localisation of the Spirochaetes and *Endozoicomonaceae* associated with *Lobophytum* and determine whether or not these bacteria are located between the coral cells or within. In addition, metagenome sequencing of the microbial community would clarify the role of Spirochaetes in the association. Microbial communities might not be a strong sex differentiation factor in *L. pauciflorum*, but as studies on other soft corals identified physiological differences between sexes (Michalek−Wagner, 2001; Fleury *et al.*, 2006), it is likely that similar characteristics exist in *L. pauciflorum*. However those physiological differences need to be verified in *L. pauciflorum* and if present the underlying molecular mechanisms behind determined. Sex−specific miRNAs have been identified in *N. vectensis* (Moran *et al.*, 2014). Sequence data that should enable identification of short RNAs for *L. pauciflorum* were also generated during my PhD studies, but their analyses are outside the scope of this thesis and remain to be analysed. Combining the developmental and microbial approaches, studying the microbial community across different life history stages starting with unfertilised eggs could offer insight in possible transmission or acquisition modes of the microbiota in *L. pauciflorum*. A combination might further reveal, which bacteria are most abundant at different developmental stages and whether the microbial community is dominated by Spirochaetes throughout development. However, before settlement stages can be investigated, appropriate cues must be identified. In addition rearing polyps without continuous water flow could favour accumulation and establishment of microbial communities that flourish in stagnant water bodies but would normally only occur in lower numbers or not at all.

Within the scope of this thesis a preliminary transcriptome assembly and a microbiome were generated for *L. pauciflorum*. In addition to these, a genome was assembled in collaboration with Dr Chuya Shinzato (Okinawa Institute for Science and Technology, Okinawa, Japan). These bodies of data, together with the preliminary analyses of developmental characteristics presented, are major steps toward a new era in soft coral biology. Although *A. millepora* or *N. vectensis* are much better characterised animals, the hope is that *L. pauciflorum* can provide additional perspectives on the evolution of developmental processes.

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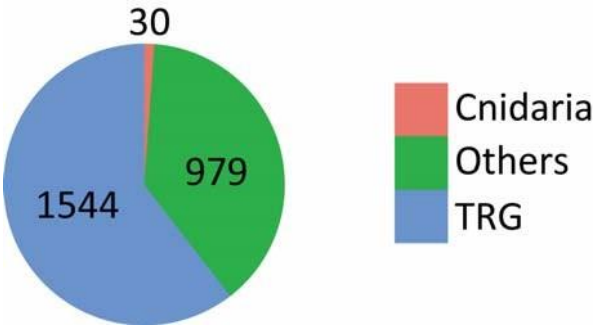
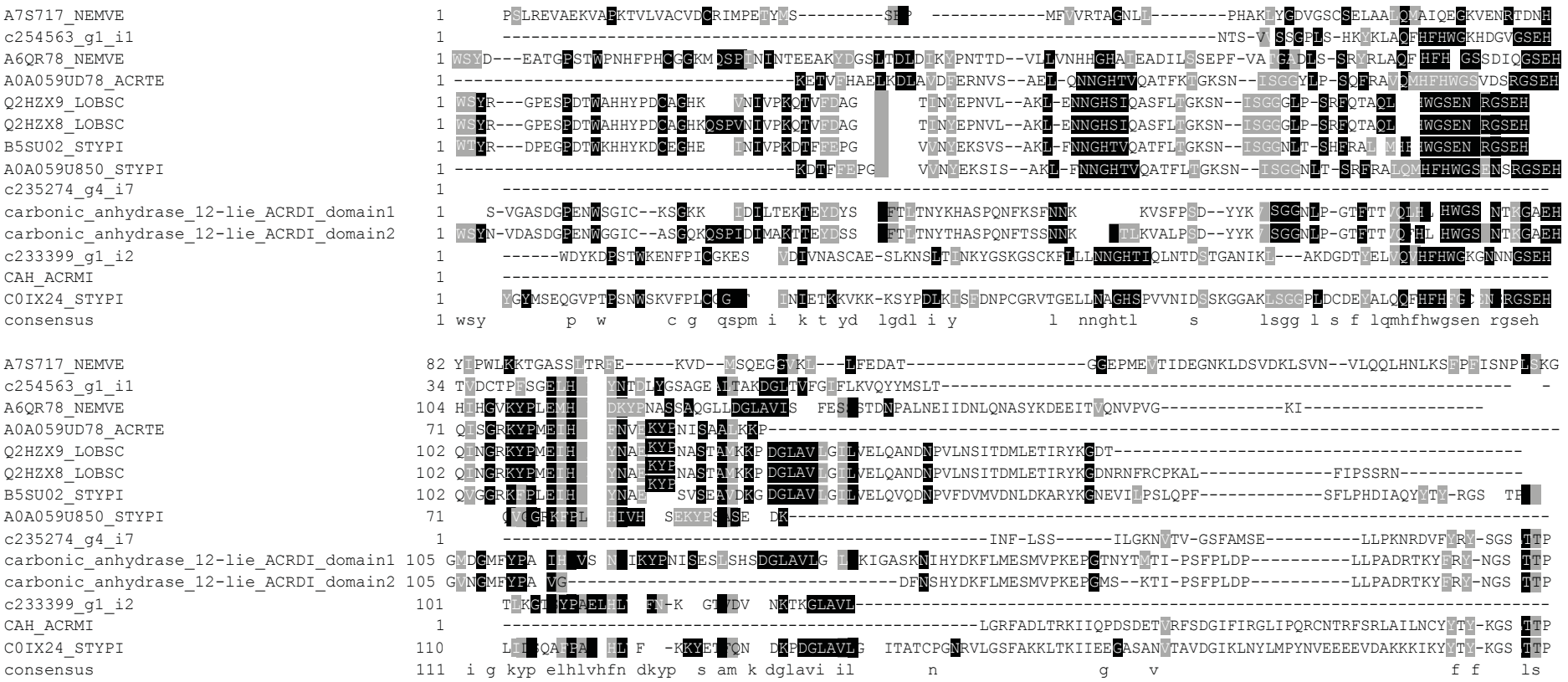
APPENDICES

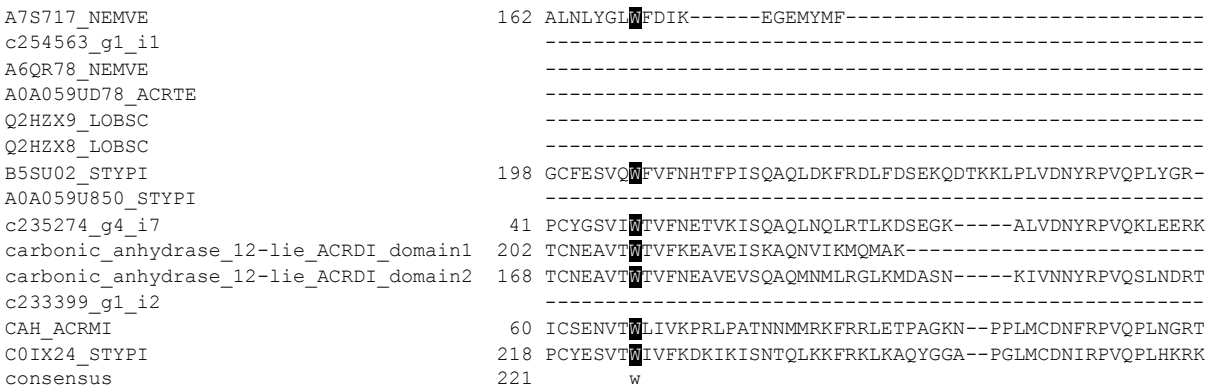
Appendix Chapter 3 Early Development

Appendix Figure 3.1 Deploying the oocyte traps in the field 2 h prior to the spawning event. A) Gently covering the soft coral colony with a trap on snorkel and B) after successfully deploying all the traps on the marked colonies, the majority of the study area is covered.

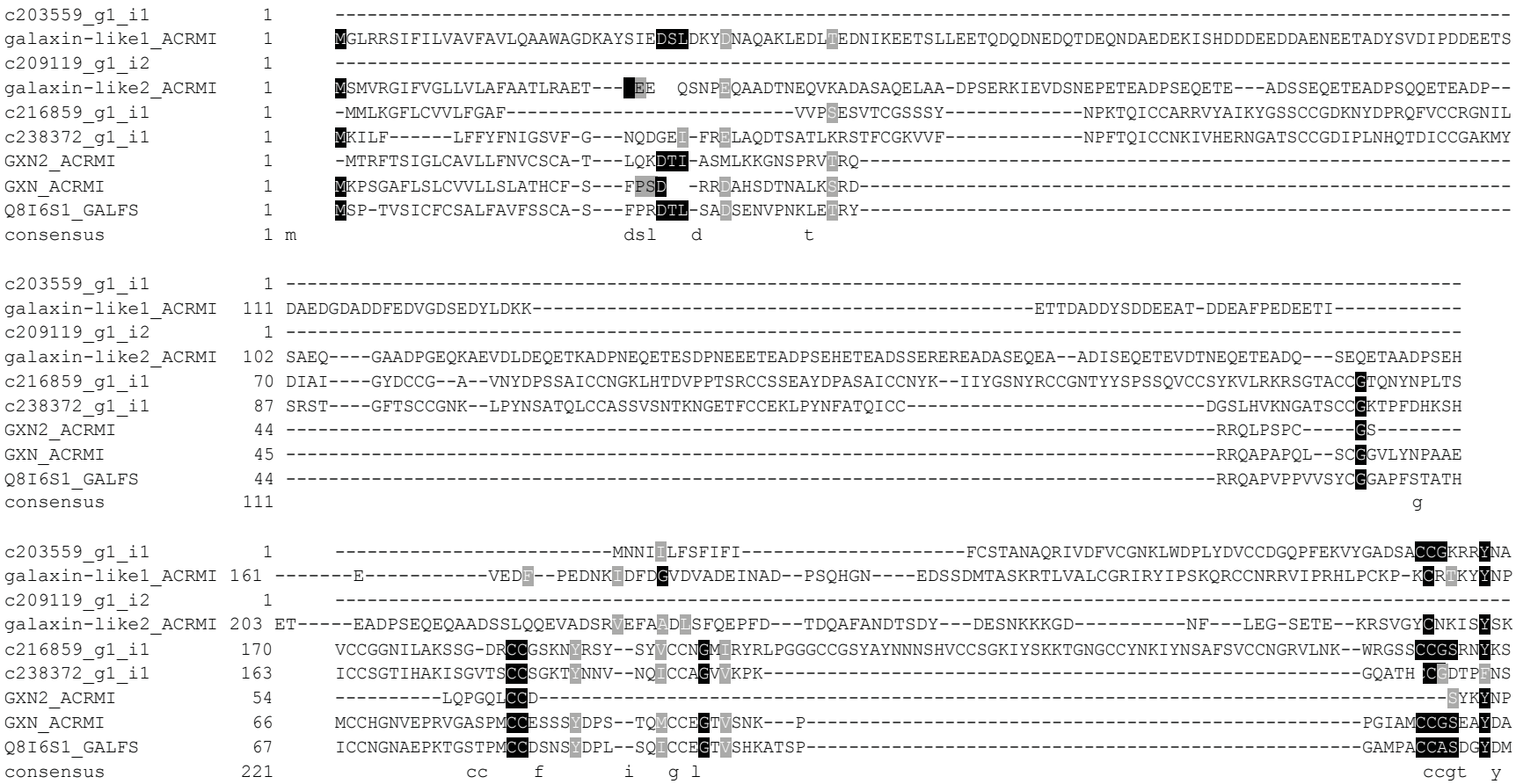


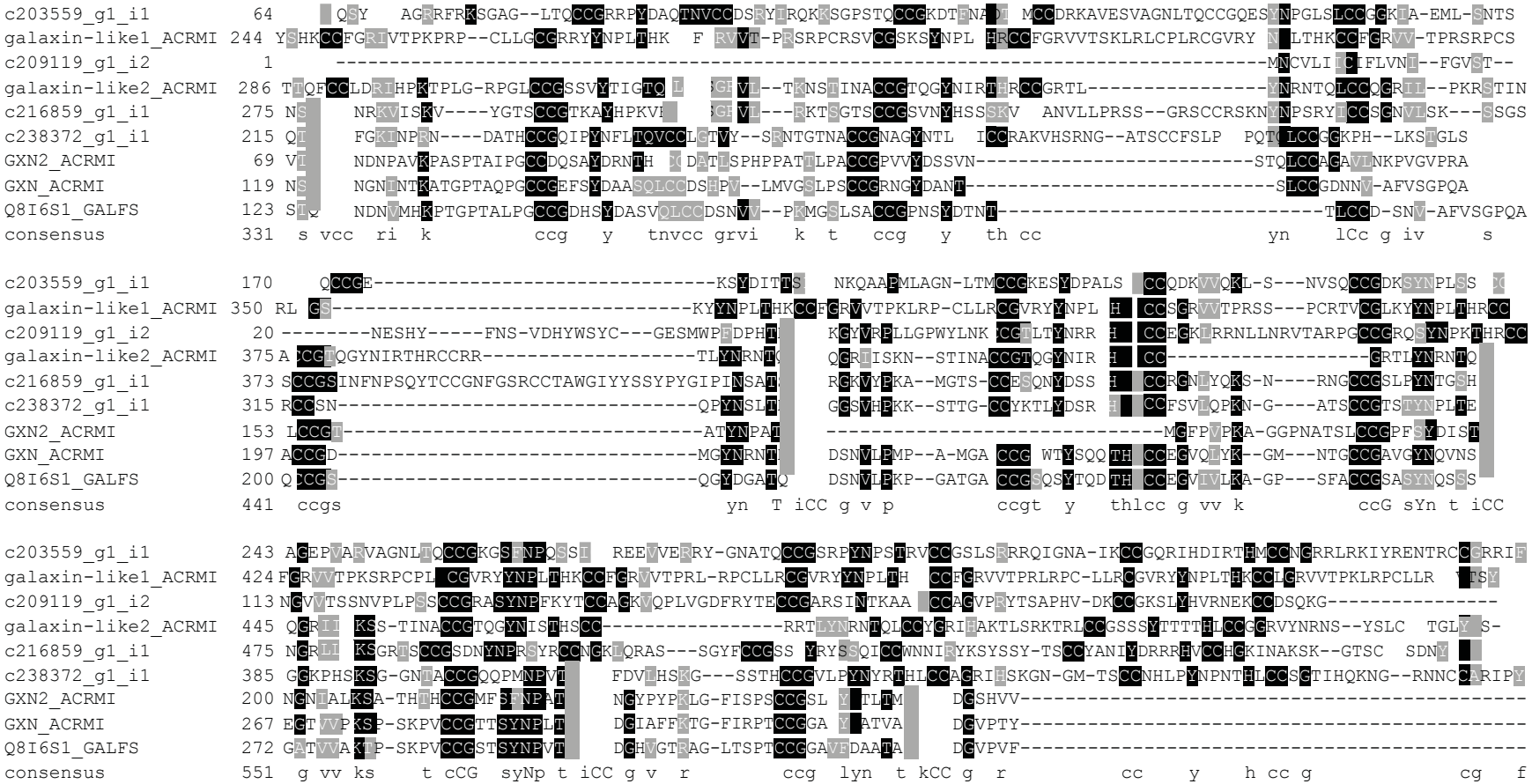
Appendix Figure 3.2 Additional pie chart to Figure 3.6 classifying the genes upregulated in planula, polyp and adult compared to the average across all stages into taxonomically restricted genes (blue – unique to *Lobophytum*), Cnidarian-specific (red – matching a database entry in Cnidaria) and other (green – matching a database entry in non-Cnidaria).


A7S717_NEMVE
c254563_g1_i1
A6QR78_NEMVE
A0A059UD78_ACRTE
Q2HZX9_LOBSC
Q2HZX8_LOBSC
B5SU02_STYPI
A0A059U850_STYPI
c235274_g4_i7
carbonic_anhydrase_12-lie_ACRDI_domain1
carbonic_anhydrase_12-lie_ACRDI_domain2
c233399_g1_i2
CAH_ACRMI
C0IX24_STYPI
consensus



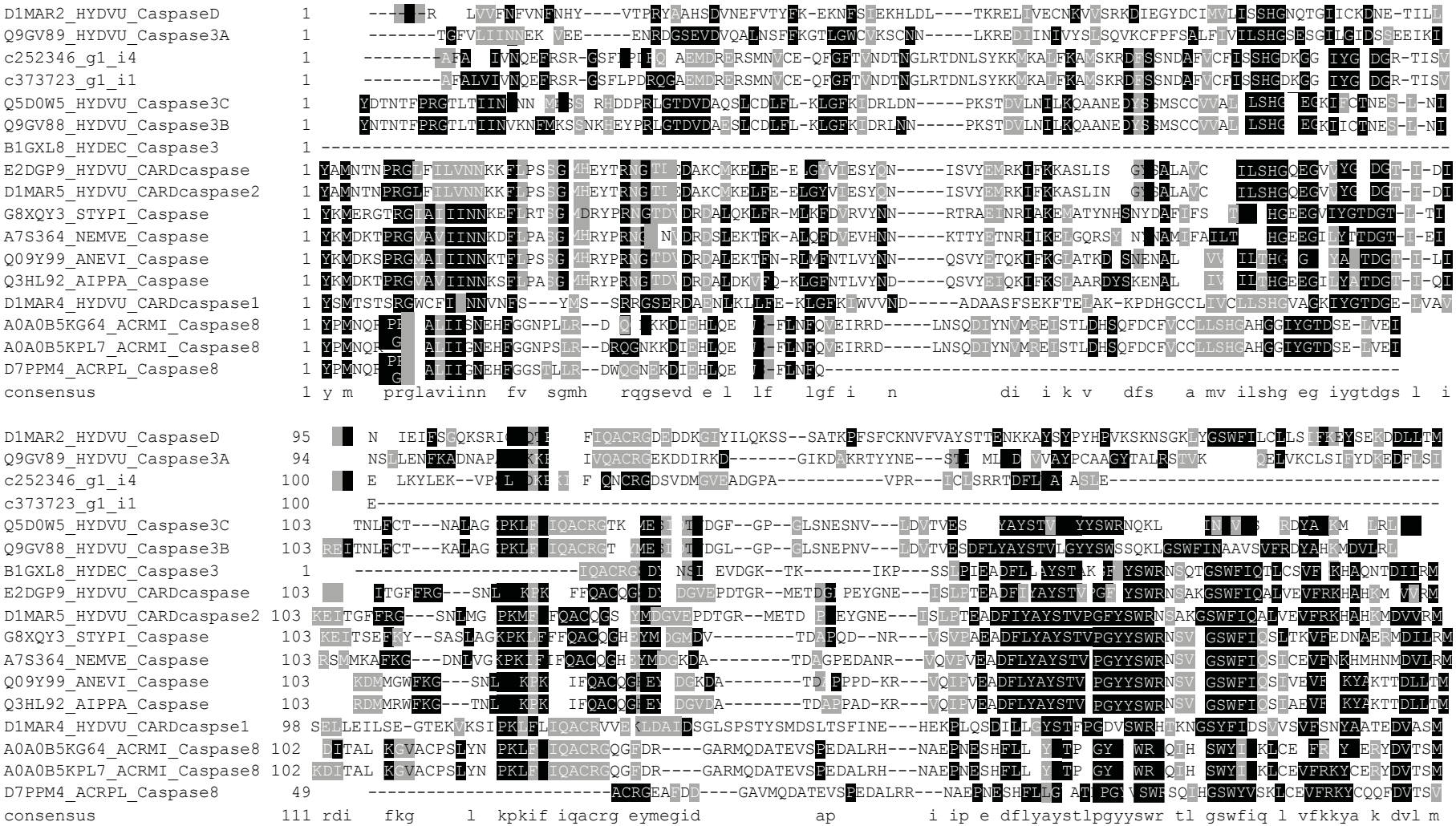
Appendix Figure 3.3 Alignment of coral carbonic anhydrase sequences,. CAH = Carbonic anhydrase, ACRDI = *Acropora digitifera*, ACRMI = *Acropora millepora,* ACRTE = *Acropora tenuis*, LOBSC = *Lobactis scutaria*, NEMVE = *Nematostella vectensis*, STYPI = *Stylophora pistillata*. Identical amino acids are highlighted in black; amino acids with similar properties are highlighted in grey.

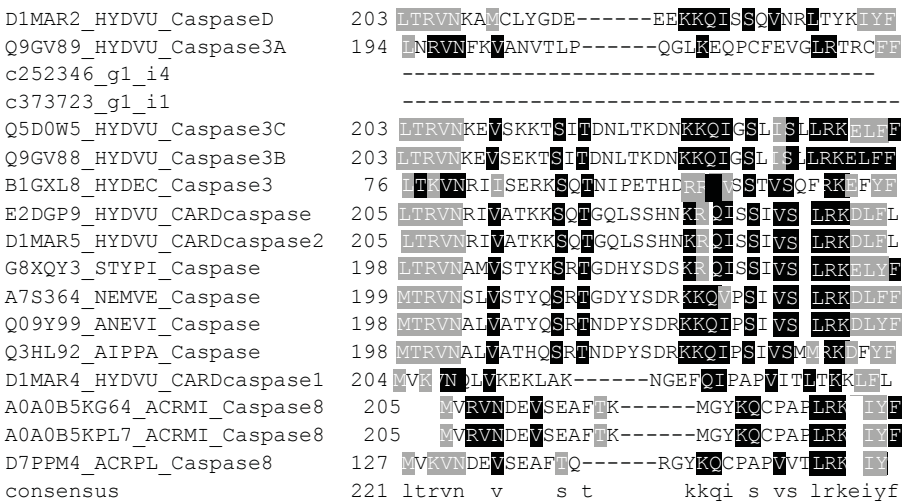

c203559_g1_i1
galaxin-like1_ACRMI
c209119_g1_i2
galaxin-like2_ACRMI
c216859_g1_i1
c238372_g1_i1
GXN2_ACRMI
GXN_ACRMI
Q8I6S1_GALFS
consensus



```
c203559_g1_i1         351 NSRRFR----------GRKKTICCANRVRY-------------------TAGFCCKGKLQARGTSCV
galaxin-like1_ACRMI   532 NPITQKCCSRRTVISKLRPCPLKCGSRYYYPTNQKCCFGHVVKATYYKPVSHRCCYGTVILKSFKCV
c209119_g1_i2         206 ---------------------------VVGSIWKDCPRKSDENVVEIY-------------------
galaxin-like2_ACRMI   535 NRRVQGCCRGRSVYTLK--RQKCCTKGVI-PSWASCYCDNGHGPYISIP-------------------
c216859_g1_i1         578 NSSTHVCCGYNILLKSR--GSSCCGNHNYDPRRKKCCYRYAYGGYYLVARSQRSCY-----------
c238372_g1_i1         487 NINTHTCCRWTIHPK----GHRC-----------------------------------------------
GXN2_ACRMI            263 ----------LITPN----QDPCANLA-----------------------------------------
GXN_ACRMI             330 ----------NV--------ASCACGLA-----------------------------------------
Q8I6S1_GALFS          335 ----------NV--------PSCACGLA-----------------------------------------
consensus             661 n          l          c a
```

Appendix Figure 3.4 Alignment of coral galaxin sequences. GXN = Galaxin, ACRMI = *Acropora millepora*, GALFS = *Galaxia fascicularis*. Identical amino acids are highlighted in black; amino acids with similar properties are highlighted in grey.





Appendix Figure 3.5 Alignment of cnidarian caspase and caspase-associated sequences. ACRMI = *Acropora millepora*, ACRPL = *Acropora palmata*, AIPPA = *Aiptasia pallida*, ANEVI = *Anemonia viridis*, HYDEC = *Hydractinia echinata*, HYDVU = *Hydra vulgaris*, NEMVE = *Nematostella vectensis*, STYPI = *Stylophora pistillata*. Identical amino acids are highlighted in black; amino acids with similar properties are highlighted in grey.

Appendix Table 3.1 Summary of over-represented genes per gene ontology (GO) term identified in uniquely and mutually upregulated contigs in 183 hpf planula (Pla), 1 wps polyp (Pol) and adult (Adu) when temporal expression is compared to average expression of all stages. GO terms are grouped by biological process (BP), cellular component (CC) and molecular function (MF).

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Adu-Pla	All
BP	Ammonium transport	-	-	-	-	-	-	7
BP	Bioluminescence	-	-	-	-	-	-	7
BP	Biosynthetic process	-	-	-	-	9	-	-
BP	Carbohydrate metabolic process	8	-	-	-	-	-	28
BP	Cell adhesion	-	-	-	17	-	-	-
BP	Cell communication	-	-	-	7	-	-	-
BP	Cell differentiation	-	-	-	-	-	-	4
BP	Cellular component organization	-	-	-	5	-	-	-
BP	Cellular iron ion homeostasis	-	-	-	-	-	-	3
BP	Cellular protein modification process	-	6	-	-	-	-	-
BP	CTP biosynthetic process	4	-	-	-	-	-	-
BP	DNA methylation on adenine	-	-	-	-	-	-	4
BP	Glutamine metabolic process	-	-	-	-	-	-	5
BP	Glutathione metabolic process	-	-	-	-	-	-	5
BP	G−protein coupled receptor signaling pathway	-	-	-	17	-	-	-
BP	Growth	-	-	-	-	-	-	10
BP	GTP biosynthetic process	4	-	-	-	-	-	-
BP	Immune response	4	-	-	7	-	-	8
BP	Ion transport	-	9	-	-	-	-	-
BP	Lipid biosynthetic process	-	5	-	-	-	-	-
BP	Lipid metabolic process	-	-	-	-	-	-	14
BP	Metabolic process	9	-	-	-	-	-	25
BP	Methylation	-	2	-	-	-	-	-
BP	Nodulation	-	2	-	-	-	-	-
BP	Nucleoside diphosphate phosphorylation	4	-	-	-	-	-	-
BP	Oligosaccharide biosynthetic process	-	2	-	-	-	-	-

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Adu-Pla	All
BP	Peptide cross−linking	-	-	-	-	-	-	6
BP	Phospholipid catabolic process	-	4	-	-	-	-	5
BP	Protein folding	3	-	-	-	-	-	9
BP	Proteolysis	-	-	-	16	5	-	66
BP	Regulation of cell growth	-	4	-	-	-	-	-
BP	Regulation of microtubule polymerization or depolymerization	-	-	-	2	-	-	-
BP	Regulation of transcription, DNA−templated	-	-	-	19	-	-	-
BP	rRNA modification	-	2	-	-	-	-	-
BP	rRNA processing	-	3	-	-	-	-	-
BP	Steroid hormone mediated signaling pathway	-	-	-	4	-	-	-
BP	Translational elongation	-	-	-	-	-	-	5
BP	Transmembrane transport	-	-	-	-	-	-	34
BP	tRNA processing	-	5	-	-	-	-	-
BP	UTP biosynthetic process	4	-	-	-	-	-	-
CC	Collagen trimer	-	-	-	-	-	-	12
CC	Cytoskeleton	-	-	-	15	-	-	-
CC	Endoplasmic reticulum	-	-	-	-	-	-	5
CC	Eukaryotic translation elongation factor 1 complex	-	-	-	-	-	-	5
CC	Extracellular matrix	-	-	-	4	-	-	5
CC	Extracellular region	-	7	-	-	-	-	22
CC	Integral component of membrane	-	-	-	45	-	-	-
CC	Keratin filament	-	-	-	-	-	-	3
CC	Membrane	15	-	-	26	-	-	80
CC	Proteinaceous extracellular matrix	-	-	-	14	-	-	-
CC	Viral capsid	-	-	-	-	-	-	5
MF	Actin binding	-	-	-	8	-	-	-
MF	Acyl−CoA dehydrogenase activity	-	-	-	-	-	-	7

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Adu-Pla	All
MF	Ammonia−lyase activity	-	-	-	-	9	-	-
MF	Ammonium transmembrane transporter activity	-	-	-	-	-	-	7
MF	Calcium ion binding	14	-	-	40	-	-	-
MF	Calcium−dependent phospholipid binding	3	-	-	-	-	-	-
MF	Carbohydrate binding	-	-	-	-	-	-	31
MF	Cysteine−type endopeptidase activity	-	-	-	-	5	-	-
MF	Cysteine−type peptidase activity	-	-	-	-	-	-	15
MF	DNA binding	-	-	-	22	5	-	-
MF	Extracellular matrix structural constituent	-	-	-	-	-	-	12
MF	Extracellular−glutamate−gated ion channel activity	-	-	-	-	-	-	9
MF	Ferric iron binding	-	-	-	-	-	-	3
MF	Gamma−glutamyltransferase activity	-	-	-	-	-	-	5
MF	G−protein coupled receptor activity	-	-	-	17	-	-	-
MF	Growth factor activity	-	-	-	-	-	-	11
MF	GTP binding	-	-	-	-	4	-	-
MF	GTPase activity	4	-	-	-	-	-	-
MF	Heparan sulfate proteoglycan binding	-	-	-	8	-	-	-
MF	Hormone activity	-	3	-	-	-	-	4
MF	Hydrolase activity	-	10	-	-	5	-	28
MF	Insulin−like growth factor binding	-	4	-	-	-	-	-
MF	Ion channel activity	-	8	-	-	-	-	15
MF	Ionotropic glutamate receptor activity	-	-	-	-	-	-	9
MF	Kinase activity	-	-	-	4	-	-	-
MF	Metal ion binding	-	13	-	-	-	-	-
MF	Metalloendopeptidase activity	-	-	-	-	-	-	26
MF	Metalloendopeptidase inhibitor activity	-	-	-	-	-	-	2

Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Adu-Pla	All
MF	Metallopeptidase activity	-	-	-	-	-	-	6
MF	Methyltransferase activity	-	8	-	-	-	-	-
MF	N−acetyltransferase activity	-	-	-	-	3	-	-
MF	Nucleic acid binding	-	-	-	-	5	-	-
MF	Nucleoside diphosphate kinase activity	4	-	-	-	-	-	-
MF	O−methyltransferase activity	-	3	-	-	-	-	-
MF	Oxidoreductase activity	11	4	-	-	-	-	46
MF	Peptidase inhibitor activity	-	-	-	-	-	-	10
MF	Peptidyl−dipeptidase activity	-	-	-	-	-	-	6
MF	Peptidyl−prolyl cis−trans isomerase activity	-	-	-	-	-	-	9
MF	Phospholipase activity	-	4	-	-	-	-	5
MF	Polysaccharide binding	-	-	-	7	-	-	-
MF	Procollagen−proline 4−dioxygenase activity	-	-	-	-	-	-	5
MF	Protein binding	-	53	-	-	-	-	-
MF	Protein dimerization activity	-	-	6	-	-	-	-
MF	Protein−glutamine gamma−glutamyltransferase activity	-	-	-	-	-	-	6
MF	Protein−L−isoaspartate (D−aspartate) O−methyltransferase activity	-	6	-	-	-	-	-
MF	Pyridoxal phosphate binding	-	-	-	-	-	-	7
MF	rRNA (adenine−N6,N6−)−dimethyltransferase activity	-	2	-	-	-	-	-
MF	rRNA methyltransferase activity	-	2	-	-	-	-	-
MF	S−adenosylmethionine−dependent methyltransferase activity	-	2	-	-	-	-	-
MF	Scavenger receptor activity	-	-	-	9	-	-	-
MF	Selenium binding	-	-	-	-	-	-	4

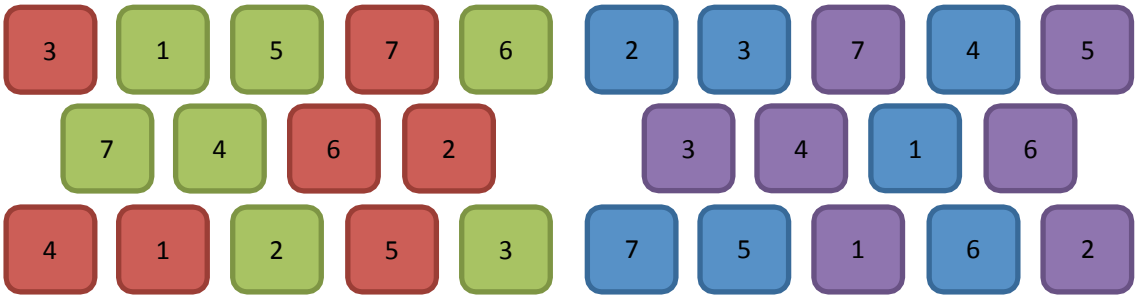
Ontology	Term	Pla	Pla-Pol	Pol	Pol-Adu	Adu	Adu-Pla	All
MF	Sequence−specific DNA binding transcription factor activity	-	-	-	8	-	-	-
MF	Serine−type endopeptidase activity	-	-	-	10	-	-	15
MF	Serine−type endopeptidase inhibitor activity	-	4	-	-	-	2	8
MF	Site−specific DNA−methyltransferase (adenine−specific) activity	-	-	-	-	-	-	4
MF	Starch binding	-	-	-	-	-	-	3
MF	Steroid hormone receptor activity	-	-	-	4	-	-	-
MF	Thyroxine 5'−deiodinase activity	-	2	-	5	-	-	-
MF	Transaminase activity	-	-	-	-	-	-	7
MF	Transferase activity	8	-	-	4	-	-	5
MF	Translation elongation factor activity	-	-	-	-	-	-	5
MF	Transmembrane transporter activity	-	-	-	-	-	-	11
MF	Transporter activity	-	-	-	-	-	-	14
MF	Triglyceride lipase activity	-	-	-	-	-	-	7
MF	Tumour necrosis factor receptor binding	4	-	-	-	-	-	8

Appendix Table 3.2 Log fold change of galaxin, CA and caspase sequences in planula, polyp and adult stages with SwissProt annotation, PFAM domains and signal peptides. Signal peptide length is given in amino acid number.

Contig_ID	Planula	Polyp	Adult	FDR	SwissProt Top hit	e-value	Pfam	e-value	SignalP
c203559_g1_i1	−2.08	6.19	3.44	7.93E−39	Galaxin 1, *Acropora millepora*, D9IQ16.1	8.00E−38			AA 1−19
c209119_g1_i1	−1.81	1.73	4.19	5.48E−29	Galaxin 1, *Acropora millepora*, D9IQ16.1	3.00E−15			
c209119_g1_i2	−1.81	1.73	4.19	5.48E−29	Galaxin 1, *Acropora millepora*, D9IQ16.1	3.00E−15			
c209119_g1_i3	−1.81	1.73	4.19	5.48E−29	Galaxin 1, *Acropora millepora*, D9IQ16.1	3.00E−15			
c216859_g1_i1	2.21	1.33	3.17	6.12E−21	Galaxin 1, *Acropora millepora*, D9IQ16.1	1.00E−35			
c216859_g1_i2	2.21	1.33	3.17	6.12E−21	Galaxin 1, *Acropora millepora*, D9IQ16.1	1.00E−35			
c216859_g1_i3	2.21	1.33	3.17	6.12E−21	Galaxin 1, *Acropora millepora*, D9IQ16.1	1.00E−35			
c238372_g1_i1	1.06	2.16	−1.24	7.62E−07	Galaxin 1, *Acropora millepora*, D9IQ16.1	1.00E−35			AA 1−17
c252346_g1_i4	−2.15	−2.62	1.85	3.43E−08	Caspase−7, *Mus musculus*, P97864.2	3.00E−31	Peptidase C14, PF00656.17	1.10E−16	
c252346_g1_i5	−2.15	−2.62	1.85	3.43E−08	Caspase−7, *Mus musculus*, P97864.2	3.00E−31	Peptidase C14, PF00656.17	1.10E−16	
c252346_g1_i7	−2.15	−2.62	1.85	3.43E−08	Caspase−7, *Mus musculus*, P97864.2	3.00E−31	Peptidase C14, PF00656.17	1.10E−16	
c252346_g1_i8	−2.15	−2.62	1.85	3.43E−08	Caspase−7, *Mus musculus*, P97864.2	3.00E−31	Peptidase C14, PF00656.17	1.10E−16	
c373723_g1_i1	−2.15	−2.62	1.85	3.43E−08	Caspase−8, *Mus musculus*, O89110.1	5.00E−07	Peptidase C14, PF00656.17	1.10E−16	
c233399_g1_i2	−1.94	3.12	3.63	8.79E−28	Carbonic anhydrase I, *Chionodraco hamatus*, P83299.1	8.00E−25	Eukaryotic−type carbonic anhydrase, PF00194.16	4.50E−34	AA 1−18
c235274_g4_i5	2.83	1.02	1.80	2.76E−12	Carbonic anhydrase XIV, *Homo sapiens*, Q9ULX7.1	5.00E−50	Eukaryotic−type carbonic anhydrase, PF00194.16	1.10E−63	AA 1−18
c235274_g4_i6	1.90	2.61	2.96	1.00E−19	Carbonic anhydrase XIV, *Homo sapiens*, Q9ULX7.1	5.00E−50	Eukaryotic−type carbonic anhydrase, PF00194.16	1.10E−63	AA 1−18
c235274_g4_i7	2.83	1.02	1.80	2.76E−12	Carbonic anhydrase XIV, *Homo sapiens*, Q9ULX7.1	5.00E−50	Eukaryotic−type carbonic anhydrase, PF00194.16	1.10E−63	AA 1−18
c254513_g4_i1	−1.11	1.10	3.53	2.48E−22	Carbonic anhydrase II, *Rattus norvegicus*, P27139.2	2.00E−21	Eukaryotic−type carbonic anhydrase, PF00194.16	5.70E−26	
c254513_g4_i2	−1.11	1.10	3.53	2.48E−22	Carbonic anhydrase II, *Rattus norvegicus*, P27139.2	2.00E−21	Eukaryotic−type carbonic anhydrase, PF00194.16	5.70E−26	

Contig_ID	Planula	Polyp	Adult	FDR	SwissProt Top hit	e-value	Pfam	e-value	SignalP
c254513_g4_i3	−1.11	1.10	3.53	2.48E−22	Carbonic anhydrase II, *Rattus norvegicus*, P27139.2	2.00E−21	Eukaryotic−type carbonic anhydrase, PF00194.16	5.70E−26	
c254563_g1_i1	−1.27	1.19	3.86	1.04E−26	Carbonic anhydrase VII, *Homo sapiens*, P43166.1	6.00E−26	Eukaryotic−type carbonic anhydrase, PF00194.16	1.30E−28	
c254563_g1_i4	−1.27	1.19	3.86	1.04E−26	Carbonic anhydrase VII, *Homo sapiens*, P43166.1	6.00E−26	Eukaryotic−type carbonic anhydrase, PF00194.16	1.30E−28	
c254563_g1_i5	−1.27	1.19	3.86	1.04E−26	Carbonic anhydrase VII, *Homo sapiens*, P43166.1	6.00E−26	Eukaryotic−type carbonic anhydrase, PF00194.16	1.30E−28	
c254563_g1_i7	−1.27	1.19	3.86	1.04E−26	Carbonic anhydrase VII, *Homo sapiens*, P43166.1	6.00E−26	Eukaryotic−type carbonic anhydrase, PF00194.16	1.30E−28	

Appendix Chapter 4 Microbiome



Appendix Figure 4.1 Experimental design of individual tanks, tanks with identical letters indicate origin of the mother colony; Number and colours describe the treatment in order to generate a unique code for each sample, green = control, red = heated, blue = acidified, purple = heated & acidified; n = 7 in each treatment.

Appendix Table 4.1 Relative abundance ± standard error of the 832 core OTUs in *L. pauciflorum* at class level according to sampling site. Letters indicate significantly different relative abundances between sampling sites that do not carry the same letter, $p < 0.05$, n = 8 for Cattle Bay, n = 5 for Little Pioneer Bay and n = 4 for Pelorus.

Class	Cattle Bay	Little Pioneer Bay	Pelorus
a-Proteobacteria	9.89 ± 3.87^{a}	2.32 ± 1.12^{a}	6.16 ± 2.70^{a}
y-Proteobacteria	$24.97 \pm 10.82^{a,b}$	43.70 ± 11.43^{a}	9.17 ± 2.74^{b}
Spirochaetes	33.04 ± 8.43^{a}	39.36 ± 10.51^{a}	65.40 ± 7.88^{b}
Others	1.22 ± 0.21^{a}	$2.13 \pm 0.95^{a,b}$	2.25 ± 0.47^{b}
Unassigned	30.87 ± 6.83^{a}	12.49 ± 4.70^{b}	$17.02 \pm 6.82^{a,b}$

Paper draft

The microbiome of the soft coral Lobophytum pauciflorum and its response to environmental stress

Wessels W[1,2], Sprungala S[2], Watson SA[2], Miller DJ[1,2], Bourne DG[3,4]

1) ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia
2) College of Public Health, Medical & Vet Sciences, James Cook University, Townsville, Queensland, Australia
3) College of Science & Engineering, James Cook University, Townsville, Queensland, Australia
4) Australian Institute for Marine Sciences, PMB 3, Townsville, Queensland, Australia

ABSTRACT

Bacteria associated with marine invertebrates are increasingly recognised as having important functional roles that benefit the host including production of antibacterial products, which exclude other harmful microorganisms and recycling of essential nutrients. This study characterised the microbiome of a gonochoric octocoral, *Lobophytum pauciflorum*, and investigated if sex-specific differences and environmental stresses influence the diversity and stability of the associated microbiome through amplicon profiling of the bacterial 16S rRNA gene. Sequences affiliated to *Spirochaetaceae* and *Endozoicomonaceae* dominated the core microbiome of *L. pauciflorum*, representing 43 % and 21 % of the community, respectively. Among the dominant class affiliations, no sex-specific differences were detected though a 2-fold higher relative abundance of unassigned sequences was found among female samples than males. These potentially novel sequences contributed to observed differences between sexes as detected by a multivariate analysis at the OTU level. Exposing *Lobophytum pauciflorum* fragments to increased temperature (31 °C), decreased pH (7.9) and a combination of increased temperature and decreased pH for 12 days did not alter the microbial community indicating that the soft coral microbiome is stable and resilient to short term environmental stress.

Key Words

Soft coral microbiome, *Spirochaetes* dominated, ocean acidification

INTRODUCTION

Tropical coral reefs are among the most diverse ecosystems on earth with hard corals (order Scleractinia) and soft corals (order Octocorallia) representing two of the major benthic communities constituting these ecosystems (e.g. Done, 1982). The majority of reef-building corals are hard corals (Veron, 1986) although some soft coral species consolidate their reef with sclerites (Schuhmacher, 1997; Jeng *et al.*, 2011). Despite lacking an exoskeleton, soft corals provide additional structural complexity for fishes and other marine invertebrates (Crossland, 1983; Dinesen, 1983), though their importance for reef ecosystems is often overlooked. Soft coral have a well-developed chemical defence mechanism, which has been the focus of much of the research into these reef inhabitants (Tursch *et al.*, 1974; Coll *et al.*, 1982; Coll *et al.*, 1986; Coll *et al.*, 1989; Coll *et al.*, 1995).

The soft coral *Lobophytum pauciflorum* (Ehrenberg, 1834) is a common species in the shallow waters of the Indo-West Pacific (Tursch and Tursch, 1982; Verseveldt, 1983; Benayahu, 2002). Like most soft corals (Kahng *et al.*, 2011), *L. pauciflorum* is a gonochoric species with dimorphic polyps, though the importance of separate sexes in this species is yet to be determined. Interestingly differences in the UV protective mycosporine-like amino acids (Michalek-Wagner, 2001) and complimentary secondary metabolite concentration (Fleury *et al.*, 2006) identified between female and male colonies, indicate the possibility for physiological differences between the sexes. Secondary metabolites were found to have cytotoxic and antimicrobial properties in this species (Zhao *et al.*, 2013), though physiological differences in secondary metabolites between different sexes have not been investigated. Sex determining mechanisms or further discrimating factors consolidating the hypothesis of the origin of the physiological differences observed, are so far unknown. If physiological differences between *L. pauciflorum* sexes exist however, they might be driven by a differentially regulated immune system. This phenomenon has been observed in bilateria, where sex-specific differences in the immune system have been identified as one possible source for physiological differences (Klein, 2000; Peng *et al.*, 2005; Love *et al.*, 2008; Nunn *et al.*, 2009). The immune system may also influence the associated microbial communities of the soft coral with an increasing number of studies recognising the importance of microbial constitutents within the coral organsim, the sum of which is termed the coral holobiont (Bosch and McFall-Ngai, 2011). Microbial communities have been well studied in hard corals; however, despite the abundance of the soft coral *L. pauciflorum*, this species has been little studied at either the organismal, molecular or microbial levels (Fan *et al.*, 2005; Yan *et al.*, 2010a; Yan *et al.*, 2010b; Yan *et al.*, 2011).

The cnidarian immune system contains homolog features to those identified in the bilaterian innate immune system (Lesser *et al.*, 2004; Palmer *et al.*, 2012). Some of those shared components are pattern recognition molecules and signal transduction pathways (Lesser *et al.*,

2004; Augustin *et al.*, 2010; Palmer *et al.*, 2012). Upon exposure to a common fungal parasite, the immune system of the Caribbean sea fan *Gorgonia ventalina* responded with differential expression of pattern recognition molecules and antimicrobial peptides amongst others (Burge *et al.*, 2013). In order to inhibit proliferation of bacteria, removal of infected cells via the apoptotic pathway is crucial. Host induction of apoptosis is the preferred mechanism for terminating the immune response following control of infection because tissue damage, an inevitable consequence of inflammation, is limited with this mode of cell death (Haslett, 1999; Kanaly *et al.*, 1999). In all metazoans, apoptosis is essential for embryonic development, immunological defence, oxidative stress and cellular as well as tissue homeostasis. In the coral relative *Hydra*, apoptosis is morphologically indistinguishable from apoptosis mediated cell death in vertebrates and invertebrates (Cikala *et al.*, 1999). In addition to the morphological similarities, two genes with strong homology to caspase-3 are expressed and the caspase-3 like enzyme is active during apoptotic cell death in *Hydra* (Cikala *et al.*, 1999). The recent description and characterisation of caspases in *Acropora millepora* (Moya *et al.*, 2016) strengthens the broad apoptotic cell death repertoire in the common ancestor for Cnidaria and Bilateria.

The term microbiome describes 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that share the body space' (Lederberg and Mccray, 2001). Scleractinia corals benefit from the microbial associates in terms of nitrogen fixation (Rohwer *et al.*, 2002; Lesser *et al.*, 2004), antibiotics production (Ritchie, 2006) and mucus recycling and food supply (Wild *et al.*, 2004). Within hard corals, studies have identified highly diverse though often conserved microbial populations associated with coral species (Rohwer and Kelley, 2004; Bourne *et al.*, 2008). During periods of environmental stress the microbiome of corals has been shown to shift either driven by a host response and/or changes in the microbiome itself (Bourne *et al.*, 2008). For example during bleaching events the coral associated microbiomes are more diverse (Bourne *et al.*, 2008) accompanied by a shift in functional composition from autotrophic to heterotrophic communities (Littman *et al.*, 2011). Vega Thurber and colleagues (2009) observed shifts from healthy-associated coral microbial community to a community often found in diseased corals under experimental stress in *Porites compressa*. Shifts in microbial community away from normal homeostasis might also trigger the innate immune response in order to fend off potential infections. Recently studies have also begun to explore the core microbiome, a subset of microorganisms that is shared by most individuals of the species with crucial functional roles, though not necessarily composed of the most abundant microorganisms (Ainsworth *et al.*, 2015). These communities may play a vital role in coral health during time of stress (Shade and Handelsman, 2012) with loss of the core microbiome contributing to reduced host fitness.

The microbiome of gorgonians has been investigated previously and members of the *y-Proteobacteria* class were identified as dominant. The majority of retrieved sequences from 16S rRNA gene profiling studies were affiliated with the genus *Endozoicomonas* (Sunagawa *et al.*, 2010; Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). This host-microbial association is potentially symbiotic due to evidence pointing at a long-term partnership with little geographic or seasonal influence (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015). Further, this association seems to be species-specific with each gorgonian species having a host specific *Endozoicomonas* ribotype even on a regional scale and potential host-dependant selection factors (La Rivière *et al.*, 2015).

In this study we investigated the microbiome of the soft coral *Lobophytum pauciflorum* and explored the potential to discriminate between male and female colonies on the microbial level. Further we investigated the effect of experimentally applied natural stressors such as elevated temperature and CO_2 to determine the acute response (1 d) of *L. pauciflorum* as well as its ability to deal with these stressors for a few days (12 d). We studied caspase activity as part of the apoptotic immune response in the coral host and the effect of both temperature and CO_2 on the microbial community profiles of *L. pauciflorum*.

MATERIALS & METHODS

Animal collection and maintenance

Two separate collections of *Lobophytum pauciflorum* colonies were undertaken in August 2013 and October 2014 from around Orpheus and Pelorus Islands (18.57° S, 146.48° E; 18.54° S, 146.49° E according to the GBRMPA permit G12/35295.1), to supply samples for the experimental analyses. For the sex-specific microbiome analyses, 17 *L. pauciflorum* colonies were sampled from three locations around Orpheus Island group (Cattle Bay, Little Pioneer Bay and Pelorus Island) and these sampling locations were one factor for the multivariate analysis. The collected colonies were brought to Orpheus Island Research Station (OIRS) and held in a 1000 L flow through tank and acclimated for one week prior to sampling of two replicate lobes from each colony. One lobe was immediately snap frozen in liquid nitrogen for total DNA extraction and the second lobe transferred into HEPES buffered seawater with 4 % formaldehyde for fixation. After taking tissue samples the colonies were returned to the ocean to a marked location, before bringing them back to the station one month later with the sex of the colonies determined by histology and/or coral spawning.

For the temperature and CO_2 stress experiments, fragments of 6 *Lobophytum pauciflorum* colonies were transported and cultured in a 1000L tank with flow through at the Marine Aquaculture Research Facility Unit at James Cook University, Townsville for further propagation. After one week of acclimation the fragments were cut into two pieces followed by

a recovery period of 3 days. The fragments were halved again so that 4 small fragments in total of each colony were obtained, which were transported back to OIRS and left to acclimate for 2 weeks prior to experimental manipulation.

Histological preparation and sex identification

Following fixation for 24 h, tissue samples were washed twice with tap water and decalcified overnight in 10 % formic acid, rinsed with water to remove the formic acid and cut for transactional and longitudinal analyses prior to storage in 70 % ethanol. Tissue samples were embedded in paraffin and three 5 µm thick sections obtained every 50 µm of tissue sample. Sections were stained with hematoxylin and eosin to facilitate gonad recognition. Gonads were identified using a 10x magnification of a compound stereomicroscope (Olympus). Presence of oocytes and spermatogonia indicated the sex of the colony as female or male, respectively. If gonads were not clearly visible in tissue sections, the sex of the colony was identified during the annual coral spawning event. 8 colonies were identified as females and 9 as males.

Experimental design for temperature and CO_2 stress

The temperature and CO_2 conditions chosen for the manipulative experimental stress were based on previous studies with the hard coral *Acropora aspera* and *A. millepora* and which resulted in documented suppression of physiological parameters (Ogawa *et al.*, 2013) and apoptosis (Moya *et al.*, 2014). For the CO_2 treatment, natural seawater in a 500 L sump tank was acidified with 100% CO_2 using a pH computer (Aquamedic AT-Control, Germany) as described in Watson *et al.* (2012). For the control treatment, a non-acidified 500 L sump tank was used. Following acidification, the seawater was heated with in-line pond titanium heaters (Weinu, Taiwan) to 32°C in two 60 L header tanks that were supplied with seawater from of the sump tanks. Each of the 60 L header tanks fed seven 3 L aquaria in which the coral fragments were placed randomly, one coral fragment per tank (Supp. Figure S1). The flowthrough into each experimental aquarium was adjusted to 250 mL/min to ensure maintenance of experimental conditions. Coral were kept at a 12/12 h day/night cycle with Sanyo lights (Sydney, Australia) connected to a timer clock.

After transferring the coral fragments into the experimental aquaria, temperature was increased in the respective 60 L header tank from 27 °C to 32 °C over a 48 h period and pH was decreased over 6 h after the temperature had reached 32 °C. Water temperature and pH_{NBS} (model MW102, Milwaukee, USA) were controlled (at least 3 times a day) and corals were monitored for visual signs of bleaching or infection. The experiment time started when stressors had reached their experimental level, of a pH of 7.9 and temperature 32 °C, respectively.

Samples for total DNA extraction and caspase activity assays were taken 1 and 12 days after the start, snap frozen in liquid nitrogen and stored at –80 °C until processing.

Total alkalinity and CO2 partial pressure

Seawater chemistry analyses were performed using water samples taken daily from the four 60 L header tanks and preserved with mercuric chloride, 0.04 % final concentration, 100 mL seawater with 40 µL saturated $MgCl_2$. Seawater chemistry was analysed following Miller *et al.* (2012). Briefly total alkalinity was determined by Gran titration with certified reference material (Batch 136, Dr A. G. Dickson, Scripps Institution of Oceanography). Total alkalinity of header tanks was used to calculate the pCO_2 in experimental tanks based on the pH and temperature measurements taken for individual tanks at time of sampling in CO2SYS (Pierrot *et al.*, 2006) using the NBS scale as pH scale and all other settings as standard (Table 1). Daily average values for salinity were downloaded from the data centre of the Australian Institute for Marine Sciences for relay pole 2 (http://data.aims.gov.au/aimsrtds/datatool.xhtml?site=9).

Total DNA extraction and microbiome sequencing

Frozen tissues samples (~ 25 mg) were ground to powder in liquid nitrogen and total DNA extracted with the DNase Blood and Tissue kit (Qiagen, Australia) following the supplier's instruction. To avoid RNA contamination, an additional RNA digest was performed according to the manual's suggestions. DNA concentration was measured using the Qubit® HS DNA assay (Invitrogen/ Life Technologies, Australia) and integrity verified on agarose gel.

To identify the microbial diversity, the V3 and V4 region of the Prokaryotic 16S ribosomal RNA gene was amplified following the 16S library preparation guide for the Illumina MiSeq system. Briefly, the V3 and V4 regions were amplified with PCR primers including the Illumina overhang adapter sequences, which resulted in a PCR product of about 500 bp. DNA samples were diluted to 5 ng μL^{-1} prior to PCR amplification. For each sample the PCR reaction was perform in triplicates. After purification, the concentration of each PCR product was determined (Qubit® HS DNA assay, Invitrogen/ Life Technologies, Australia) and triplicates for each sample were combined at an equimolar concentration of 2 nM. Illumina adapter sequences and dual-index barcodes were added to each PCR mix with the Nextera XT Index kit (Illumina, USA) in a second PCR reaction. The prepared libraries were pooled at an equimolar ratio of 2nM after purification. Libraries were sequences on MiSeq at James Cook University using paired 300-bp reads and MiSeq v3 reagents (Illumina, USA). As an internal sequencing control, the library pool was spiked with 10 % PhiX control (Illumina, USA).

The sequencing run was monitored over BaseSpace and data were directly uploaded from the MiSeq machine. Sequencing resulted in approximately 1.86 million reads passing the filter with on average 38,000 reads per sample, ranging from 16,000 – 75,000 reads.

Microbiome analysis

Sequence data were available as demultiplexed reads on BaseSpace. The first 16 bases were trimmed of the forward reads and the first 18 of the reverse reads to remove the primer sequences. Further downstream analysis was conducted in QIIME (Version 1.9) (Caporaso et al., 2010) and built-in functions. Prior to de novo OTU picking using mothur with 97 % sequence similarity (Schloss *et al.*, 2009; Schloss *et al.*, 2011), chimeric sequences were removed using USEARCH61 (Edgar, 2010). Cleaned sequences were aligned against the SILVA database (SILVA SSU Release 119, July 2014) (Quast *et al.*, 2013).

Sequence data from sex-specific and stress experiments were analysed separately. The sex-specific microbiome sequencing resulted in 121369 OTUs with 2,263,516 counts. For the stress experiment a total of 34,947 OTUs were observed with 1,988,523 counts. However the counts varied from 81,204 to 218,374 counts per sample in the sex experiment and from 3,248 to 119,032 in the stress experiment. For statistical analysis, only OTUs with more than 15 counts in total were considered, which reduced the number observed OTUs to 2,578 and 3,509, respectively.

To gain an insight into the most abundant ribotyptes associated with *L. pauciflorum*, sequences of the 20 most abundant OTUs were extracted from the each data set and blasted (blastn) (Altschul *et al.*, 1997) against the NCBI database. The core microbiome was analysed using the *compute_microbiome* function in QIIME as suggested by Ainsworth *et al.* (2015). Based on previous studies (Philippot *et al.*, 2013) a representation of 51 % was chosen for the core microbiome analyses of the sex and the stress experiment. This implies if an OTU was not present in at least 51 % of the samples than it was considered as individual variability of colonies.

Sequencing read counts were normalised using the relative log expression method proposed by Anders and Huber (2010) in edgeR (Robinson *et al.*, 2010). Following normalisation, counts were standardised by species and site and a multivariate correspondence analysis was performed with vegan (Oksanen, 2013, 2015). A multivariate correspondence analysis was chosen instead of a principle component analysis as the data did not meet the requirements for neither normality nor homogeneity of variance. The 20 most abundant OTUs were extracted from normalised read counts and used to generate a heat map with gplots (Warnes *et al.*, 2009; Warnes, 2015) and RColorBrewer (Neuwirth, 2011). Box and CA plots

were produced using ggplot2 (Wickham, 2009). To generate the Venn diagrams the R package limma (Ritchie *et al.*, 2015) was used.

Apoptotic cell death

To investigate the host response to simulated environmental stressors increased temperature, low pH and the combination of increased temperature and low pH, the occurrence of apoptotic cell death was studied. Frozen coral tissue samples (~ 15 mg) were homogenized in 1.5 mL (1:100 (w v^{-1})) hypotonic extraction buffer (25 mM HEPES, 5 mM MgCl2, 1 mM EGTA and protease inhibitors (complete protease inhibitor cocktail, Roche, Australia; 1 pellet in 10 mL buffer)) using a FastPrep®-24 (MP Biomedicals, Australia) at program 3, twice with a 5 min interval on ice until tissue was completely homogenized. Subsequently samples were centrifuged (5810-R, Eppendorf) for 15 min and 13,000 xg at 4 °C. Caspase enzyme activity was measured using a Caspase-Glo 3/7 assay kit (Promega, Australia). Briefly 50 µL of supernatant and 50 µL reagent were mixed in a white-walled 96-microwell plate (NUNC F96) and incubated at 25 °C for 1 h. The light intensity emitted by each sample was measured in a GENios Pro Microplate Reader by Tecan (Männerdorf, Switzerland) in the luminescence mode. In each plate a sample blank containing 100 µL sample and reagent blank containing 50 µL buffer and 50 µL reagent were measured. To control for reagent quality on subsequent days and comparability between different kits, one reference sample was prepared as 0.5 mL aliquots and deep frozen at – 80 °C. One of these aliquots was measured once a day. Caspase activity was measured in relative light units (RLU) and normalized to protein concentration. Caspase activity was calculated as RLU mg $protein^{-1}$. Protein concentration was determined using the Qubit® Protein assay (Invitrogen/ Life Technologies, Australia).

RESULTS

The microbial community associated with *Lobophytum pauciflorum*

A total of 2578 OTUs were identified to be associated with *Lobophytum pauciflorum,* derived from 2,161,800 sequencing reads with stringent quality checking and considering only OTUs with 15 or more reads. The core microbiome, which represented sequences retrieved from at least 51% of samples of *Lobophytum pauciflorum*, was composed of 832 OTUs. Despite the smaller number of identified OTUs in the core microbiome the the bacterial diversity patterns were highly similar to microbial patterns when all the data was investigated, and therefore futher analysis and interpretation is based only on this core microbiome. The microbiome was dominated by the class of *Spirochaetes* and the family of *Spirochaetaceae*

representing on average 43 % of the retrieved sequences across all samples (Table 2). The second most abundant taxonomic class was the *γ-Proteobacteria* which constituted approximately 27 % of the recovered sequences. Within the *γ-Proteobacteria*, 23 % of the sequences were related to the family of *Endozoicimonaceae*. *α-Proteobacteria* related sequences accounted for 7 % of recovered microbial 16S rRNA gene sequences across all samples though these fell within a diverse array of different family groups, with *Rickettsiaceae* being the most abundant family representing 4 % of the assigned OTUs. Interestingly despite stringent sequence quality checking and comparisons to the SILVA microbial database, a high percentage of retrieved sequences (~ 22 %) could not be assigned (Table 2).

A direct comparisons between male and female colonies demonstrated similar core microbiome profiles for the different soft coral sexes at the class taxonomic affiliation level (Table 2). The male microbial community was however characterised by a slightly higher relative abundance of *Spirochaetes*-related sequences though this was not significantly different to the female community profile ($p > 0.1$) (Table 2). At the family level, OTUs affiliated with the *Rhodobacteraceae* were about 4 times more abundant in male than in female corals, though this difference was not significant ($p > 0.05$). The female microbiome was characterised by an almost two times higher relative abundance of sequences that were unassigned, 29 % compared to 16 % in males colonies, though this difference was not significant ($p > 0.05$) (Table 2). The relative abundance of *Rickettsiaceae*-related sequences were also twice as high in female soft corals than in males, though the difference was not significant ($p = 0.27$), due to high standard errors within each sex. A multivariate correspondence analysis of the core mirobiome structure with constraints for both sex and sampled colony explained 35.12 % of the variation. The three sampling locations (Cattle Bay, Little Pioneer Bay and Pelorus Island) significantly ($p < 0.05$) separated along the first and second constraint correspondence axis, which explained 16.67 % and 9.24 % of the variation, respectively, of the association between samples and microbial community (Figure 1). The male and female microbial communities were significantly different along the second constraint correspondance axis, $p < 0.05$ (Figure 1).

The 20 most abundant ribotypes made up approximately 83 % of the total core microbiome based on their normalised counts (Figure 2A). OTU 1 was closely related to an *Endozoicomonas* sp. sequence first identified in a marine sponge and represented 25% of all retrieved sequences (Figure 2A). Of the 20 most abundant OTUs, 5 were closely related to *Endozoicomonas* sp. and accounted for 29.5 % of the core microbiome community (Figure 2A). OTU 2 was related to a *Spirochaetes* sp. sequence recovered from marine sediments and constituted 23% of the retrieved sequences from this study. Other OTUs 3, 4 and 5 were also closely related to *Spirochaetes*-affiliated sequences and together accounted for approximately 39 % of the community structure (Figure 2A). Among the 20 most abundant OTUs were also 3

sequences, OTU 10, 14 and 17, that were closely associated with a *Cytophaga* sp. BHI80-3 sequence and accounted for 4 % of the relative abundance of the core microbiome (Figure 2A). Although *Alteromonadaceae* were not represented among the most abundant families, OTU 7 which represented 2 % of the retrieved sequences, was closely related to an *Alteromonadales* sp. extracted from a marine sponge (Steinert *et al.*, 2014). *Ricksettiaceae* related sequences (OTU 8) were the most abundant family within the *α-Proteobacteria*, though only represented 2 % of retrieved sequences (Figure 2A). Interestingly, OTU 2 was highly abundant within all *Lobophytum* samples, whereas the other OTUs showed a colony specific variation in relative abundance (Figure 2A).

Effects of environmental stress on the microbial community and host response

Microbial community

The response of the microbial community associated with *Lobophytum pauciflorum* to environmental stress conditions over a period of 12 days including; (1) a 4 °C increase of water temperatures, (2) decrease in pH of 0.2 pH units and (3) a combination of increased temperature and decreased pH was investigated. Sequencing the 16S rRNA of samples from all 4 experimental conditions, allowed the identification of 3509 OTUs derived from 1,886,381 sequencing reads recovered from *Lobophytum pauciflorum* samples with stringent quality checking and considering only OTUs with 15 or more reads. The core microbiome at 51 % representation comprised 249 OTUs dominated by *Spirochaetes* related sequences, assigned to the family of *Spirochaetaceae* which represented 34 % of the retrieved sequences (Table 2). The second most abundant class in the core microbiome were the *γ-Proteobacteria* (22 % of retrieved sequences) dominated by *Alteromonadaceae*- and *Enterobacteriaceae*-related sequences (6 % and 5 % of the sequences, respectively) though *Endozoicomonaceae*-related sequences were also relatively abundant (4 % of retrieved sequences, Table 2). *Endozoicomonaceae*-related sequences increased by roughly 4 % in samples taken after 12 days of exposure to environmental stressors while relative abundance of *Alteromonadaceae*-related sequences decreased by about 4 % (Table 2). *Vibrionaceae* related sequences contributed to a minor extent (2 %) to the microbial profile of *L. pauciflorum*. The relative abundance of sequences assigned to this *γ-Proteobacteria* family increased 3-fold between day 1 and day 12 of the experiment ($p < 0.05$), similar to the increase of *Endozoicomonaceae*-related sequences (Table 2). Approximately 15 % of all sequences were associated with *α-Proteobacteria* and the *Rhodobacteraceae*-related sequences (10 % of the retrieved sequences) the most abundant at this family level. Only 5%, of the sequences were assigned to *Flavobacteriia*-related sequences, though the relative abundance was about 3 times lower after 12 days of exposure to environmental conditions than after 1 day ($p < 0.05$, Table 2).

Approximately 15 % of the sequences could not be assigned to known 16S rRNA sequences in the database.

Correspondence analysis provided no evidence for the sampling time point or environmental stress conditions of temperature, pH or combined temperatture and pH influencing the *Lobophytum* microbiome (Figure 3A). Further being kept in a flow through system and neither of the experimental conditions did alter the microbial community, relative microbial abundances at class level were similar in samples taken either 1 day or 12 days after the experiment commenced (Table 2). The clustering pattern within the microbial communities samples was however explained by the colony that the microbiome profiles were originally sampled from (Figure 3B). The explanatory power of the correspondence analysis was limited with the first and second correspondence axis explaining only 13 % and 11 % percent of the variation, likely due to the high similarity of the microbial community between all samples. However small shifts in the minor groups of ribotypes associated with individual colonies appeared to be the principal driver in the seperation of samples of the correspondence analysis (Figure 3B).

Host response

The 20 most abundant OTUs of the core microbiome accounted 51 % of the total retrieved sequence abundance (Figure 2B). The three most abundant OTUs, OTU 1 – 3, with 6 – 7 % relative abundance each were associated with *Spirocheata* sp.-related sequences and inclusive of OTU7 accounted for a total of 22 % of the microbial community profiles. Two ribotypes associated with a *Cytophaga* sp. BHI80-3 sequence contributed 4 % of the community. The relative abundance of *γ-Proteobacteria* and *α-Proteobacteria* related sequences both represented approximately 6% of retrieved sequences (Figure 2B). *γ-Proteobacteria* were represented by *Enterobacteraceae*- and *Alteromonadaceae*-related sequences, while *α-Proteobacteria* were only represented by *Rhodobacteraceae*-related sequences. Colony specific OTUs were also observed in this experiment, with OTU 7 and 14 being abundant in colony 1 and OTU 2 and 4 being more abundant in colonies 4 and 5. Of the 20 most abundant OTUs identified in this stress experiment 8 OTUs (OTUs 1, 2, 3, 5, 7, 8, 11, and 14) were also identified among the 20 most abundant OTUs in the sex experiment and assigned to the same sequences (Figure 2A, B).

Caspase activity, as marker for host response to stress and as part of the apoptotic immune response system, was measured in the soft coral fragments subjected to temperature and CO_2 stress, though did not vary between sampling time points, 1 d vs 12 d, or between environmental stress conditions (Supp. Figure 4.6 A). Within treatment and time point variation was higher than between treatments and time points (Supp. Figure 4.6 A). In addition, the

variability between colonies/genotypes was greater than the impact of the environmental stress, making the stress response of the host seem to be rather driven by each colony than by the stresses applied (Supp. Figure 4.6 B). A linear model could not explain these differences, potentially due to missing information about other physiological factors or sex.

DISCUSSION

The microbial community associated with *Lobophytum pauciflorum*

The microbial communities of soft corals are poorly understood though likely important within a functioning healthy colony, like those of other marine invertebrates (Rohwer and Kelley, 2004; Bourne *et al.*, 2008). The microbial profiles of *L. pauciflorum* were consistent across two separately conducted experiments in this study, highlighting the dominant abundance of *Spirochaete*- and *Endozoicomonas*-related sequences and providing a baseline understanding of the microbial communities associated with *Lobophytum*. Abundant *Spirochaete*-related sequences have been found in other coral microbiome studies such as the red coral *Corallium rubrum* from the Mediterranean Sea (van de Water *et al.*, 2016) and scleractinian corals transplanted in close proximity to a high effluent fish culture in the Philippines (Garren *et al.*, 2009). Low abundances of *Spirochaetes* were also detected on a site specific dependant pattern in *Stylophora pistillata* colonies at the GBR (Kvennefors *et al.*, 2010) and in the gorgonian coral *Plumarella superba* from the Aleutian islands (Gray *et al.*, 2011). Further *Spirochaetes* were identified as a small constituent of the endosymbiotic core microbiome in three mesophotic hard corals (Ainsworth *et al.*, 2015). In terrestrial insects, *Spirochaetes* are commonly observed in the hindgut of termites establishing an obligate symbiosis that has coevolved (To *et al.*, 1978; Berlanga *et al.*, 2007). The localisation of this dominant bacterial group associated with *L. pauciflorum* tissues is unknown, though as observed with other invertebrates, it may be within the tissue layers rather than as epibionts on the outside of tissues. *Lobophytum* sp. have also been shown to exhibit high antimicrobial properties due to secondary metabolites produced by the coral host (Yan *et al.*, 2010a; Yan *et al.*, 2010b; Yan *et al.*, 2011) which may further indicate an internal rather than external pattern of colonisation of the *Spirochaetes*. *Spirochaetes* have also been suggested to have an important role in structuring the microbial community of *C. rubrum* by secretion of antibiotics (van de Water *et al.*, 2016). However further studies that localise this community within the *L. pauciflorum* tissues using fluorescent *in situ* hybridisation approaches targeting this specific group with oligonucleotides probes is warranted.

γ-Proteobacteria were the second most abundant class of sequences associated with *L. pauciflorum* (~ 26% relative abundance, Table 2) and the majority of sequences within this class were affiliated with the *Endozoicimonaceae* (~ 23 % relative abundance; Table 2).

Endozoicomonaceae are a family within the *γ-Proteobacteria* that is commonly found in association with hard and soft corals but also other marine invertebrates (Bayer *et al.*, 2013b; Bourne *et al.*, 2013; Nishijima *et al.*, 2013; Lema *et al.*, 2014a; La Rivière *et al.*, 2015). Previous genome sequencing of symbiotic *Endozoicomonas* strains suggested a functional role in nitrate cycling and a symbiotic relationship with the host (Neave *et al.*, 2014). An intimate relationship of host endoderm cells and aggregations of *Endozoicomoas* were identified for the Red Sea corals *Stylophora pistillata* and *Pocillopora verrucosa* also suggesting an endosymbiotic relationship (Bayer *et al.*, 2013b; Neave *et al.*, 2016). Further, recent studies suggest a close coevolution of *Endozoicomonas* strains and gorgonian species (Bayer *et al.*, 2013a; La Rivière *et al.*, 2015) and therefore similar symbiotic relationships with this bacterial family and *L. pauciflorum* may exist. However specific *in-situ* localisation studies combined with host-microbe functional analyses are required to confirm these relationships.

Among the *α-Proteobacteria*, *Rickettsiaceae*-related sequences were identified as the most abundant (4 % relative abundance, Table 2). In scleractinian corals, cell-associated microbial aggregates were found to be *Rickettsia*-related and were suggested to play an important role as facultative secondary symbionts (Work and Aeby, 2014). In other invertebrates such as the whitefly, *Rickettsia* sp. were found in gut and follicle cells, suggesting an endosymbiotic relationship between fly and bacteria (Gottlieb *et al.*, 2006). *Rickettsia* were identified to be transmitted vertically but also horizontally within the population, suggesting that infection leads to an advantageous phenotype in the whitefly though the exact function that these bacteria have in the biology of the whitefly remains to be determined (Gottlieb *et al.*, 2006). A contrasting example from the pea aphid revealed that *Rickettsia*-infection had negative effects on some components of the host fitness when compared to *Rickettsia*-eliminated strains from the same genetic background (Sakurai *et al.*, 2005). In *Lobophytum* and many other marine invertebrates, the role of *Rickettsiaceae*-related sequences needs to be further explored. It is possible that this bacterial family is also localised among cell-associated microbial aggregates as in scleractinian corals (Work and Aeby, 2014), however their functional role within the octocoral microbiome needs to be further investigated potentially through a metagenome sequencing approach.

Lobophytum colonies were further associated with low abundant *Pseudoalteromonadaceae* and *Vibrionacaea*-related sequences, 0.7 % and 0.5 %, respectively (Table 2). Both *Pseudoalteromonas* sp. and *Vibrio* sp. associated sequences are widely recovered from scleractinian coral microbiome studies and *Vibrios* in particular often associated with compromised coral health (Bourne *et al.*, 2008; Vega Thurber *et al.*, 2009; Séré *et al.*, 2013). However *Vibrionaceae*-related sequences may also be a normal constituent of the coral microbiome and Ainsworth *et al.* (2015) suggested that this family might rather be associated

with the surface mucus. Due to the antimicrobial activity of the secondary metabolites discovered in *Lobophytum* sp. (Zhao *et al.*, 2013) it is possible that members of the *Vibrionaceae* recovered among the sequences in *L. pauciflorum* are also associates with the soft coral mucus. In *Pocillopora meandrina* planulae *Pseudoalteromonas* strain HIMB1276 were found to be associated with cells of the outermost ectoderm possibly involved in settlement which might indicate that *Pseudoalteromonas* sp. play a role in the induction of settlement and metamorphosis (Apprill *et al.*, 2012). Tetrabromopyrrole isolated from *Pseudoalteromonas* might induce settlement in *A. millepora* highlighting the potential importance for *Pseudomonadaceae* during settlement in scleractinian corals (Tebben *et al.*, 2011). Though the association might also have a beneficial role in adult corals due to the high abundance recovered in adult *A. millepora* (Littman *et al.*, 2009), the low abundance in *Lobophytum* recovered in this study suggests that these bacteria might only be epibionts associated with the soft coral mucus or particles. In addition to members of *Spirochaetes* and *Endozoicomonas*, members of 5 families with a relative abundance below 5 % were detected (Table 2) suggesting that the microbial diversity in *Lobophytum* is low, similar to findings in *C. rubrum* where the core microbiome is composed of only 12 OTUs (van de Water *et al.*, 2016).

At class and family level, the microbial profiles associated with female and male colonies were highly consistent and the 20 most abundant OTUs that were classified taxonomically also displayed no differences in relative abundance between sexes. Multivariate correspondence analysis at the OTU level however did reveal a significant difference between sampling sites and sexes of *Lobophytum* colonies ($p < 0.05$, Figure 1). The difference between sampling sites was mainly driven by a varying relative abundance of *γ-Proteobacteria* and *Spirochaete*-related sequences, however replication for sampling sites was not evenly distributed and therefore this patterns should be viewed cautiously (e.g. 8 fragments from Cattle Bay and 4 from Pelorus, Appendix Table 4.1). The sex-specific difference might have been driven by OTUs that were not assigned to any known bacterial 16S rRNA sequence, with approximately 30% of sequences in female colonies unassigned verses 16% in male colonies. Different microbial abundances between inshore and mid-shelf sampling sites were detected in *A. millepora* where microbial communities from inshore reef corals were dominated by *γ-Proteobacteria*-related sequences while in offshore reef coral microbiomes *Bacilli-*, *α-Proteobacteria*-related sequences were more abundant (Lema *et al.*, 2014b). The three sampling sites chosen for this experiment on *L. pauciflorum* can all be considered as inshore reefs and seperated by less than 7 km. Considering the high overall similarity of the individuals and the low explanatory power of the correspondance analysis it is likely that the differences detected are rather driven by colony specific differences such as different genotypes (Figure 1, Figure 2A, Appendix Table 4.1). Similarly limited detection of significant responses in

differential gene expression studies in *Acropora millepora* has been linked to high individual variability (Bay *et al.*, 2009; Seneca *et al.*, 2010; Bertucci *et al.*, 2015) highlighting the restrictions of sequencing experiments with different genotypes. Though we did not see any difference in the dominant communities of the microbiome between male and female soft corals, sampling at different times of the year, for example during gametogenesis periods, might provide a different picture. In the current study, samples were taken 1 month prior to spawning when gametogenesis was complete, and therefore temporal sampling would be beneficial.

Effects of environmental stress on the microbial community and host response

Subjecting *L. pauciflorum* fragments to different environmental conditions including increased seawater temperature and lower pH did not shift the microbial community structure over an experimental period of 12 days (Table 2). Measurement of caspase activities, a marker for host response to stress as part of the apoptotic immune response system, also showed no significant changes under the experimental conditions over the course of the experiment. These results indicate that *L. pauciflorum* demonstrates high resilience to short term environmental stress events at both the host and microbiome levels. In contrast, Gorgonian species subjected to 4 – 16 °C higher temperature for a short period of time (1 – 4 h) were found to increase expression of heat shock proteins suggesting an upregulation of protective mechanisms as a stress response and potential for development of tolerance (Wiens *et al.*, 2000; Kingsley *et al.*, 2003). Studies on stress responses of scleractinian corals have also shown sensitivity to pH and temperature stress (1.4 units below and 5 °C above ambient) stress in adult colonies of *Porites compressa* where microbial community shifts from a healthy community to one that is often found in diseased corals (Vega Thurber *et al.*, 2009). Juvenile *A. millepora* subjected to elevated CO_2 (1.5-fold increase) for a 'prolonged', 9 d, period on the other hand showed the capacity to acclimate to elevated CO_2 conditions via suppression of apoptosis among others (Moya *et al.*, 2014). A high variability between alcyonacean species in susceptibility to bleaching was found during the mass bleaching event at the GBR in 1998 (Goulet *et al.*, 2008a). A moderate bleaching susceptibility for the family of Alcyoniidae, to which *L. pauciflorum* belongs (Goulet *et al.*, 2008a), together with a potentially similar physiological capacity to induce fast protein protection from denaturation or thermal deactivation under heat stress (Morimoto *et al.*, 1996; Nathan *et al.*, 1997) observed in gorgonians (Wiens *et al.*, 2000; Kingsley *et al.*, 2003), could explain the resilience of *L. pauciflorum* to the environmental stressors applied in this study. An unaltered apoptotic activity in *L. pauciflorum* might hence also reflect the capacity of soft corals to withstand CO_2 stress for a short period of time.

Despite no large shifts in the microbiome between the sex and the stress experiments, there were some small shifts observed including lower relative abundance of

Endozoicomonaceae-related sequences. However the lower relative abundance in the stress experiment is potentially due to culturing of sampled fragments in a recirculating system for 2 months prior to conducting the experiment. Nevertheless OTUs affiliated with *Endozoicomonaceae* were common and still represented 4 % in the microbiome of the coral fragments in the stress experiment. Transferring sponges from the wild into an aquaculture system induced a significant shift in microbial communities between wild and cultured sponges (Mohamed *et al.*, 2008). Notably the abundance of *α*- and *γ-Proteobacteria* was reduced in sponges kept in culture for up to 2 years though the shift occurred after 6 months of culturing. Returning the coral fragments to OIRS and an flowthrough system appeared to have reversed this effect as *Endozoicomonaceae*-related sequences increased over the course of the experiment, for a full 'return' of the initial 'wild' microbiome the stress experiment was probably not conducted long enough. Shifts in microbial communities in response to changing environmental conditions *in aquaria* as well as *in situ* were also observed in scleractinian corals (e.g. Bourne *et al.*, 2008; Vega Thurber *et al.*, 2009) and similar patterns are likely in *L. pauciflorum.*

The clustering patterns of the microbial profiles in the stress experiment were explained by the fragment origin, i.e. the colony the fragment derived from, similar to observations of the sex experiment supporting the idea that different genotypes are the main drivers for the differences detected. The colony-specific microbial profiles were in line with individual environmental parameters, a common observation when working with marine invertebrates. To overcome the problem of limited detection and statistical power, future studies should aim to increase the variety of genotypes from different locations but also a high number of replicates (3 – 5) for each genotype. There are few microbiome studies on octocorals and therefore these habitats provide the potential for the discovery of yet undescribed sequences from a novel environment. Sequencing errors may also be a factor in the inability to assign taxonomy, though OTUs were only considered if 15 or more reads were clustered and it is unlikely that errors would disproportionately affect sequences recovered from female colonies in the sex experiment. Further explorations of the taxonomic origin of these sequences are required potentially through fluorescent *in situ* hybridisation and metagenome sequencing approaches. This study provided a baseline understanding of the microbial community associated with the gonochoric soft coral *Lobophytum pauciflorum* and its susceptibility to environmental stress focusing on the microbial community of the holobiont and not differentiating between different coral tissues. Future studies on *Lobophytum* using these techniques will allow the localisation of *Spirochaete*- and *Endozoicomonas*-related sequences within the soft coral tissue and help identification as ecto- or endosymbiont. Successful localisation of associated microbiota within *L. pauciflorum* will enable comparison to localisation in scleractinian corals. Secondary

metabolites of *Lobophytum* sp. have been described to have antimicrobial properties (Zhao *et al.*, 2013) it is therefore possible that the detected differences between sampling sites are due to particles attached to the outside of the collected fragments rather than different internal tissue communites. Future studies should also investigate different tissue microhabitats, which might reveal interesting differences at a colony or tissue level. The importance of rare OTUs was highlighted by Ainsworth and colleagues (2015), when analysing the core microbiomes of different scleractinan coral tissues where they found that bacterial phylotypes with a potentially important endosymbiotic function were of low abundance in the holobiont community (< 5%) but very abundant in the endosymbiotic community (>20 %) (Ainsworth *et al.*, 2015). To clarify this function, metagenome sequencing will enable the detection of possible metabolic functions looking at gene predictions together with additional information on taxonomic affiliations. Overall, this study highlights that the microbial community of *L. pauciflorum* is consistent between sexes and resilient to short term environmental stresses.

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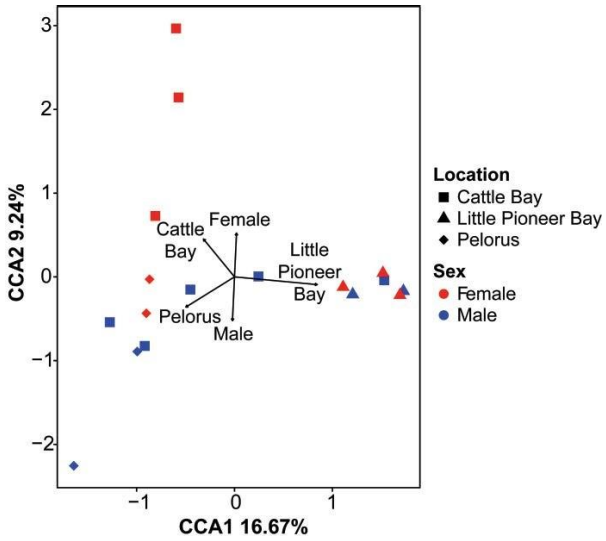
Tables and Figures

Figure 1 Correspondence analysis of OTUs comprising the sex core microbiome. Percentages in the axis titles represent the percentage described by the axis. Squares represent colonies from Cattle Bay, triangles colonies from Little Pioneer Bay and diamonds for colonies from Pelorus. n = 832 OTUs, n = 8 for female samples, n = 9 for male samples.

Figure 2 Taxa affiliation of the 20 most abundant OTUs of the sex core microbiome. Colour intensity of the heat map represents the normalised counts for the each OTU in each sample. A) n = 8 females and 9 males. B) n = 7 – 8 samples for each colony

Figure 3 Correspondence analysis the core microbiome in *L. pauciflorum* fragments subjected to temperature and CO_2 stress. Percentages in the axis titles represent the percentage described by the axis, n = 249 OTUs. (A) Squares denote sampling 1 day and triangles sampling 12 days after experimental stress conditions were established. Experimental stress conditions are differentiated by grey shades: Control in lightest grey, high temperature in light grey, high CO_2 in medium grey and combination of high temperature and high CO_2 in dark grey. n= 5 – 6 samples for each environmental stress per time point. (B) Each colony is described by its individual grey shade, n = 7 – 8 samples for each colony.


CCA2 9.24%
CCA1 16.67%
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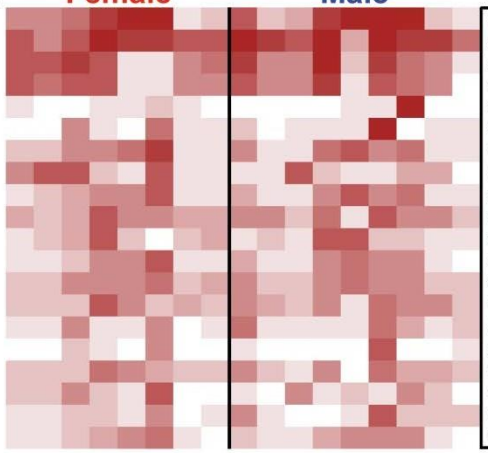
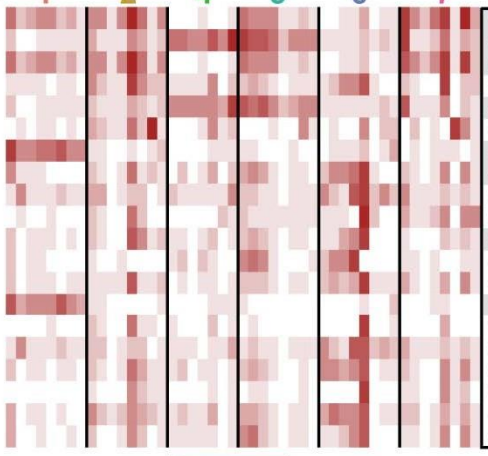
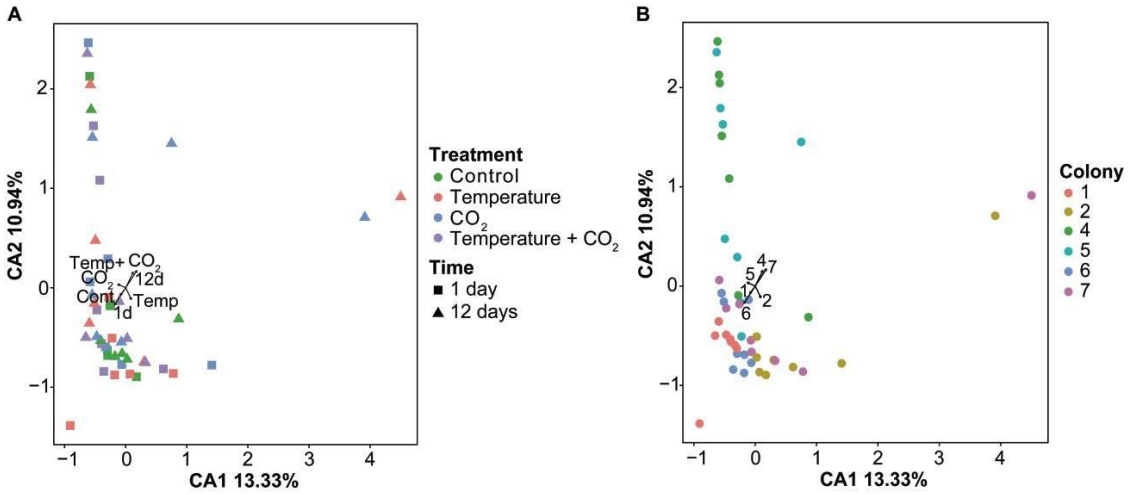


	Relative abundance	86%	Nearest relative (Acc. Number -% similarity)	Class	Biological source	OTU length (bp)
OTU1		25%	*Endozoicomonas sp.* (JX442229 - 96%)	γ-Proteobacteria	Marine sponge (unpublished)	284
OTU2		23%	*Spirochaetales sp.* (AJ620502 - 91%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU3		7%	*Spirochaeta sp.* (AY337319 - 90%)	Spirochaetes	*Spirochaeta sp.* M6 (unpublished)	284
OTU4		5%	*Spirochaeta litoralis sp.* (NR_104732 - 86%)	Spirochaetes	*Spirochaeta litoralis* (unpublished)	279
OTU5		5%	*Spirochaetales sp.* (AJ620502 - 93%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU6		3%	*Spiroplasma sp.* (KF840734 - 89%)	Mollicutes	Sheep (unpublished)	284
OTU7		2%	*Alteromonadales sp.* (KF282361 - 95%)	γ-Proteobacteria	Marine sponge (Steinert *et al.*, 2014)	284
OTU8		2%	*Orientia tsutsugamushi* (AF479303 - 91%)	α-Proteobacteria	*Orientia tsutsugamushi* strain TC586 (unpublished)	284
OTU9		2%	*Endozoicomonas sp.* (JX442229 - 97%)	γ-Proteobacteria	Marine sponge (unpublished)	279
OTU10		2%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU11		1%	*Francisella hispaniensis* (KT281843 - 96%)	γ-Proteobacteria	Blood culture (unpublished)	284
OTU12		1%	*Endozoicomonas sp.* (JX442229 - 98%)	γ-Proteobacteria	Marine sponge (unpublished)	279
OTU13		1%	*Endozoicomonas montiporae* (NR_116609 - 97%)	γ-Proteobacteria	Scleractinean coral (Yang *et al.*, 2010)	284
OTU14		1%	*Cytophaga sp.* (AJ431238 - 94%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU15		1%	*γ-Proteobacteria* sp. (FN398075 - 98%)	γ-Proteobacteria	Giant archaea (Muller *et al.*, 2010)	284
OTU16		1%	*Spiroplasma sp.* (KJ470109 - 91%)	Mollicutes	River prawn (unpublished)	280
OTU17		1%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU18		1%	*α-Proteobacteria* sp. (DQ486492 - 89%)	α-Proteobacteria	Dinoflagellate (unpublished)	284
OTU19		1%	*Sagittula sp.* (KP297965 - 99 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpublished)	284
OTU20		1%	*Endozoicomonas sp.* (KJ573540 - 96%)	γ-Proteobacteria	Marine sponge (unpublished)	284

B



	Relative abundance	48%	Nearest relative (Acc. Number -% similarity)	Class	Biological source	OTU length (bp)
OTU1		7%	*Spirochaeta sp.* (AY337319 - 90%)	Spirochaetes	*Spirochaeta sp.* M6 (unpublished)	284
OTU2		7%	*Spirochaetales sp.* (AJ620502 - 91%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU3		6%	*Spirochaeta litoralis sp.* (NR_104732 - 86%)	Spirochaetes	*Spirochaeta litoralis* (unpublished)	279
OTU4		3%	Bacterium SCSIO13079 (KJ802185 - 100%)	Bacteria	Coral reef (unpublished)	284
OTU5		3%	*Cytophaga sp.* (AJ431238 - 95%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU6		3%	*Marinifilum sp.* (KF650765 - 96%)	Bacteroidia	Marine Sediment (unpublished)	283
OTU7		2%	*Spirochaetales sp.* (AJ620502 - 93%)	Spirochaetes	Marine Sediment (unpublished)	284
OTU8		2%	*Sagittula sp.* (KP297965 -99 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpblished)	284
OTU9		2%	*Enterobacter sp.* (KR779514 - 100%)	γ-Proteobacteria	Rabbitfish (unpublished)	284
OTU10		2%	*Lysinibacillus parviboronicapiens* (NR_041589 - 86%)	Bacilli	Soil from experimental field (Miwa *et al.*, 2009)	284
OTU11		2%	*Sagittula sp.* (KP297965 -98 %)	α-Proteobacteria	*Sagittula sp.* JLT9 (unpblished)	284
OTU12		1%	*Flavobacterium sp.* (FM162878 - 96%)	Flavobacteriia	Coastal bacterioplankton (unpublished)	280
OTU13		1%	*Alteromonas macleodii* (KP074899 - 99%)	γ-Proteobacteria	Seawater (unpublished)	284
OTU14		1%	*Cytophaga sp.* (AJ431238 - 92%)	Cytophagia	*Cytophaga sp.* BHI80-3 (unpublished)	279
OTU15		1%	*Lyngbya cf. confervoides* (AY599507 - 96%)	Oscillatoriophycideae	Lyngbya cf. confervoides VP0401	284
OTU16		1%	*Klebsiella pneumoniae* (CP006659 - 100%)	γ-Proteobacteria	Klebsiella pneumoniae ATCC BAA-2146 (Hudson *et al.*, 2014)	279
OTU17		1%	*Rhodobacteraceae sp.* (KM279016 - 99%)	α-Proteobacteria	*Coccolithus braarudii* (unpublished)	284
OTU18		1%	*Verrucomicrobia sp.* (CP010904 - 85%)	Verrucomicrobia	Hypersaline microbial mat (unpublished)	284
OTU19		1%	*Rhodobacteraceae sp.* (EF629844 - 100%)	α-Proteobacteria	Flowthrough aquaculture system (Mohamed *et al.*, 2008)	284
OTU20		1%	*γ-Proteobacterium sp.* (FJ952816 - 93%)	γ-Proteobacteria	Scleractinean coral (Rypien *et al.*, 2010)	284


A
CA2 10.94%
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CA2 10.94%
CA1 13.33%
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Table 1 Summary of sea water chemistry including mean values (± SD) for temperature, pH, total alkalinity and *p*CO2 for each experimental treatment over the course of the experiment. Average salinity was 35.51 ± 0.40 psu.

Treatment	**Temperature (°C)**	**pH_{NBS}**	**Total Alkalinity (µmol $kgSW^{-1}$)**	***p*CO_2 (µatm)**
Control	27.5 ± 0.24	8.11 ± 0.02	2319 ± 10.2	502.8 ± 41.41
Temperature	30.9 ± 0.15	8.08 ± 0.02	2323 ± 4.5	542.3 ± 32.45
CO_2	27.4 ± 0.28	7.94 ± 0.01	2323 ± 13.8	779.7 ± 24.70
Temperature + CO_2	31.1 ± 0.17	7.91 ± 0.02	2325 ± 7.3	869.9 ± 38.34

Total alkalinity was measured in the four header tanks for each treatment condition. Temperature and pH were measured daily in experimental tanks of each experimental treatment. These daily measured parameters were used to estimate *p*CO_2 in CO2SYS.

Table 2 Summarised bacterial community of the microbiome of *L. pauciflorum* of two individually conducted experiments.

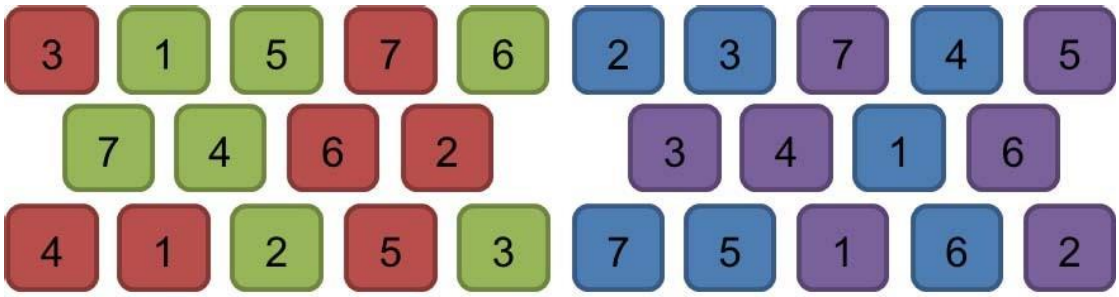
		Sex experiment			Stress experiment		
Class	**Family**	**Relative % sequence abundance**	**Female**	**Male**	**Relative % sequence abundance**	**1 day**	**12 days**
Bacteroidia	*Marinilabiaceae*	0	0	0	2.35 ± 1.10	0.28 ± 0.15	4.51 ± 2.17
Flavobacteria	*Flavobacteriaceae*	± 0.00	0.01 ± 0.00	0.01 ± 0.01	4.92 ± 1.07	7.33 ± 1.78[a]	2.41 ± 0.90
α-Proteobacteria	*Rhodobacteraceae*	1.13 ± 0.41	0.45 ± 0.18	1.72 ± 0.71	9.96 ± 1.62	10.26 ± 2.11	9.66 ± 2.54
	Rickettsiaceae	4.19 ± 2.04	5.86 ± 3.35	2.71 ±2.52	0	0	0
γ-Proteobacteria	*Alteromonadaceae*	0.14 ± 0.03	0.17 ± 0.05	0.11 ± 0.02	5.54 ± 1.02	7.20 ± 1.78	3.80 ± 0.85
	Endozoicimonaceae	22.70 ± 6.70	23.13 ± 10.01	22.31 ± 9.57	4.15 ± 1.43	1.97 ± 0.75	6.42 ± 2.76
	Enterobacteriaceae	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	4.53 ± 1.71	5.82 ± 3.10	3.19 ± 1.40
	Francisellaceae	1.50 ± 0.55	1.57 ± 0.68	1.43 ± 0.88	0.24 ± 0.10	0.23 ± 0.16	0.25 ± 0.11
	Pseudoalteromonadaceae	0.68 ± 0.37	0.18 ± 0.10	1.08 ± 0.69	0	0	0
	Vibrionaceae	0.44 ± 0.20	0.25 ± 0.12	0.62 ± 0.37	1.89 ± 0.54	0.80 ± 0.36[a]	3.03 ± 1.01
Spirochaetes	*Spirochaetaceae*	42.51 ± 6.00	35.05 ± 8.81	49.15 ± 8.00	34.04 ± 5.22	31.83 ± 6.79	36.34 ± 8.10
Others		4.49 ± 1.00	3.94 ± 0.92	4.99 ± 1.74	17.23 ± 1.90	16.83 ± 2.46	17.66 ± 2.96
Unassigned		22.21 ± 4.20	29.34 ± 7.13	15.85 ± 4.05	15.15 ± 2.90	17.45 ± 4.62	12.75 ± 3.49

Mean relative abundance ± standard error of the OTUs contributing to the core bacterial community structure of at family level, and distribution of relative abundances A) between female and male colonies and B) between sampling time points, 1 day and 12 days. [a] highlights significantly different relative abundances between period of exposure to experimental stress factors 1 day and 12 days, $p < 0.05$, n = 832 OTUs for sex experiment, n = 249 OTUs for stress experiment, n = 8 female and 9 male soft coral colonies, n = 24 fragments for 1 day exposure and 23 fragments for 12 days exposure.

Supplemental materials

Supp. Figure S1 Experimental design of individual tanks and colony fragments. Tanks with identical letters indicate origin of the mother colony. Number and colours describe the treatment in order to generate a unique code for each sample, green = control, red = heated, blue = acidified, purple = heated & acidified; n = 7 in each treatment.

Supp. Figure S2 Caspase activity as marker for host response to environmental stress and part of the apoptotic immune response system. Mean caspase activity in relative light units (RLU) per mg protein for (A) each experimental condition and sampling time and for (B) each colony of origin with standard error in *L. pauciflorum*. Light grey bars represent samples taken after 1 day and dark grey bars samples taken after 12 days under experimental conditions. Grey shades stand for the colony of origin (A) or the different environmental conditions (B). Squares and triangles in (B) denote the sampling time point of 1 day and 12 days, respectively. n = 6 – 8 samples for the combination of experimental condition and time point and colony of origin.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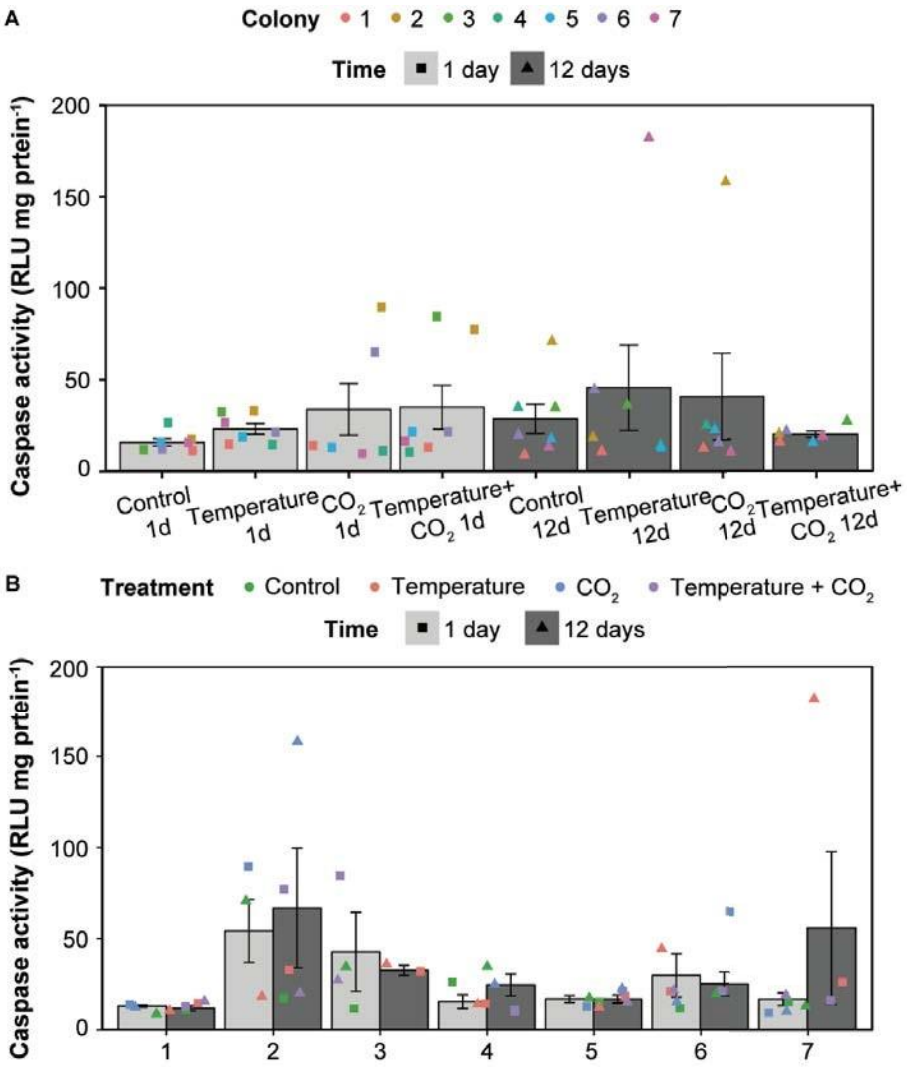
A
Colony 1 2 3 4 5 6 7
Time 1 day 12 days
Caspase activity (RLU mg prtein-1)
200
150
100
50
0
Control 1d
Temperature 1d
CO2 1d
Temperature+ CO2 1d
Control 12d
Temperature 12d
CO2 12d
Temperature+ CO2 12d
B
Treatment Control Temperature CO2 Temperature + CO2
Time 1 day 12 days
Caspase activity (RLU mg prtein-1)
200
150
100
50
0
1
2
3
4
5
6
7

Supp. Table S1 Site dependent bacterial community profile.

Class	Cattle Bay	Little Pioneer Bay	Pelorus
α-Proteobacteria	9.89 ± 3.87[a]	2.32 ± 1.12[a]	6.16 ± 2.70[a]
γ-Proteobacteria	24.97 ± 10.82[a,b]	43.70 ± 11.43[a]	9.17 ± 2.74[b]
Spirochaetes	33.04 ± 8.43[a]	39.36 ± 10.51[a]	65.40 ± 7.88[b]
Others	1.22 ± 0.21[a]	2.13 ± 0.95[a,b]	2.25 ± 0.47[b]
Unassigned	30.87 ± 6.83[a]	12.49 ± 4.70[b]	17.02 ± 6.82[a,b]

Relative abundance ± standard error of the 832 core OTUs in *L. pauciflorum* at class level according to sampling site. Letters indicate significantly different relative abundances between sampling sites that do not carry the same letter, $p < 0.05$, n = 8 for Cattle Bay, n = 5 for Little Pioneer Bay and n = 4 for Pelorus.