To Sebastian…

Cellular Mechanisms of Coral Calcification

Thesis submitted by
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in September 2009

Thesis submitted in partial fulfillment of the requirements for the Degree of **Doctor of Philosophy** in the Department of Biochemistry and Molecular Biology at James Cook University of North Queensland

"Nunc qouque curaliis eadem natura remansit, duritiem tacto capiant ut ab aere;
quodque vimen in aequore erat, fiat super aequora saxum"

The *Metamorphoses.* (IV). Publius Ovidius Naso (43 BC – 17 AD)

"A pesar que parte de la naturaleza del coral permanece, endurece en contacto con el aire; lo que vigoroso dentro del mar fue, fuera del agua en roca se convertira".

Translation by Alejandro Reyes

"Although some of its characteristics remain, coral hardens in contact with air; what was vigorous in the sea becomes like a rock out of water".

Translation by Alejandro Reyes and David Miller

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Alejandro Reyes-Bermudez

ACKNOWLEDGEMENTS

First I would like to thank my supervisor Prof. David Miller for the opportunity to work in his lab and learn the basic molecular biology techniques that allowed me to start my PhD thesis. Thanks David for your support during these years and for introducing me to coral cellular biology. I would also like to thank Prof. James Burnell for the chance to work in his lab while waiting for my scholarship as well as for his vast knowledge of metabolic pathways. Thanks Jim for all your laboratory assistance -all that looking over my shoulder really polished my lab skills-. I thank Prof. David Yellowlees for the support during the scholarship application process. Prof. Bette Willis for bringing the marine biology and fieldwork expertise to Magnetic Island. Dr. Madeleine van Oppen among other things for teaching me how to set up a PCR reaction and Dr. Kirsten Heimann for allowing me to work in her lab with my coral larvae and teaching me how to do fluorescence microscopy.

I would like to thank Dr. Eldon Ball for having me in Canberra at ANU and introducing me to some of the old calcification literature and showing me how to properly photograph coral embryos. Dr. David Hayward for his friendship, lunch-time soccer, endless lab tricks and pump master skills. I thank Dr. Lauretta Grasso for assistance with microarray experiments at ANU and fieldwork in Orpheus Island collecting larvae for microarray experiment. I thank Zhiyi Lin and others members of Eldon's Lab at ANU for 1) characterizing a third galaxin gene which strengthen the galaxin paper/chapter, 2) repeating and validating the expression patterns of the galaxing genes shown in the thesis and 3) editing and reviewing the first draft of the galaxin chapter.

I thank assistant professor Monica Medina at UC Merced for the opportunity to work in her lab and learn how to do microarray analysis and for editing the first drafts of chapter 1 and 2. Thanks Monica for the chance to work in Mexico with Caribbean coral species and for that "Alejandro no sabes escribir" it really helped me to improve my writing. I thank Mickey DeSalvo for our *Montastrea* array paper, friendship and support during my time in Merced. Thanks Mickey for editing and commenting on the first draft of the Calmodulin chapter. I thank Dr. Chris Voolstra for annotating the sequences used in the microarray chapter. I thank all members of the Medina Lab at

UCM for all their help and assistance during my time at UCM. I also would like to thank Prof. Denis Allemand and Dr. Sylvie Tambutté from the Monaco Scientific center for commenting on a first draft that served as a templete for chapter 1.

I would like to thank present and past members of the Miller laboratory for sharing the day-by-day endeavours in this cold and quiet environment ideal for protein purification. I think this is the closest I get to an Antartic expedition. I thank Nikki Hislop and Danielle De Jong for sharing protocols during the early stages of my PhD. Chuya Shinzato for sharing the latest findings in transcription factors expression patterns, Akira Iguchi for the fertilization lecture during the 2006 coral spawning, the Miso soups and the blues performances. I thank Victor Hugo Beltran Ramirez for "Los Gallos clandestinos" y el melodrama Mejicano. I hope the time machine you are working on takes you places "güey". I also want to thank Prof. Michael Ridd and Prof. Beverley Glass for our weekly meetings that helped David to read my thesis and Barbara Pennach for all the assistance with scholarship and postgraduate matters.

Finally I would like to thank my family and friends especially Kirsty Hewitt for the support and patience over the PhD years, Lene Noreng for sharing her liver pate and Norwegian caviar here in Townsville and for having me in Oslo on my way to Bergen, Jillian Grayson for helping me to format the thesis, pivot table tutorials and diving experiences. I thank the undersea explorer for an ACRS student award that allowed me to observed in Osprey reef the exuberant repercussions of coral calcification. I thank NGED for a laboratory exchange award that allowed me travel to ANU to learn how to do microarray hybridisations and JCU for a JCUVC scholarship. Finally, I would like to thank the Latin American truck drivers, without them I would have not made it to Australia and a big thanks to "los chamanes de la Amazonia" without them I would have not had the energy and focus to study the cellular mechanisms of coral calcification.

Townsville, August 6th 2009.

ABSTRACT

Calcification is a basic process by which the controlled deposition of calcium salts provides the basis of supportive structures in both vertebrate and invertebrate organisms. While vertebrates use calcium phosphate in their skeletons, many invertebrates use calcium carbonate in the form of calcite and/or aragonite. It is assumed that vertebrates and invertebrates calcifying structures evolved separately and represent discontinuous evolutionary systems. However, the fact that some components of the calcification repertoire such as carbonic anhydrases and calcium homeostasis mechanisms have an ancestral origin suggests that while extracellular components of calcifying matrices have been recruited independently in different taxa, basic cellular ion homeostasis and transport mechanisms are likely to be conserved.

It has been suggested that calcification in corals evolved from an ancestral calcium sink mechanism. As well as being a successful evolutionary strategy, the evolution of scleractinian exoskeletons has had important ecological consequences, as corals constitute the framework of tropical reef ecosystems. Corals are the only anthozoan group in which settlement and tissue reorganization during metamorphosis correlates with the deposition of a juvenile skeleton. While the oral ectoderm retains the columnar organization seen in planulae, the aboral ectoderm is transformed from a columnar epithelium into a squamous calcifying cell type known as the “calicoblast”. This cell type secrets and matures an organic matrix that regulates the nucleation of calcium carbonate crystals.

This study focuses on metamorphosis and the early stages of calcification in the scleractinian coral *Acropora millepora,* and focuses on proteins of the galaxin family and EF-hand calcium sensors as potential regulators of larval morphogenesis and skeleton deposition. In parallel, primary cell cultures were established from five key developmental stages and gene expression microarray analysis was used to investigate changes in gene expression during metamorphosis and post-settlement life. Although the results reported here reveal a high level of complexity, they will facilitate the elucidation of the gene networks involved in processes such as lineage specific cell differentiation and juvenile calcification.

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CHAPTER 1. GENERAL INTRODUCTION.

1. 1. Biomineralization.

Biomineralization is the formation of minerals by living organisms ranging from metal crystals in bacteria to mineral structures in unicellular and multicellular eukaryotes (Wang *et al.,* 2008; Wilt, 2005). Animal calcification is one of many biomineralization processes by which controlled deposition of calcium salts produce supportive structures in both vertebrate (i.e. bones, teeth) and invertebrate species (i.e. molluscan shells, coral skeletons). Calcification is a fundamental process that occurs in multiple animal groups ranging from basal metazoans such as sponges and corals to deuterostomes such as echinoderms and chordates (Marin *et al.,* 2008). While vertebrates use calcium phosphate in the form of hydroxyapaptite to build their skeletons, many invertebrates use calcium carbonate in the form of calcite and/or aragonite (Fukuda *et al.* 2003; Taylor *et al.,* 2008).

It is thought that vertebrates and invertebrates calcifying structures evolved separately and constitute discontinuous evolutionary systems (Marin *et al.,* 2008). The fact that calcifying matrix proteins in both sea urchins (Livingston *et al.,* 2006) and vertebrates (Kawasaki and Weiss, 2006) belong to small gene families and are found tightly clustered in their respective genomes suggests 'recent' independent origins by gene duplication (Kawasaki and Weiss, 2006). The finding that 85% of the secreted proteins involved in shell formation in the mollusc *Haliotis asinina* are unknown novel proteins (Jackson *et al.,* 2006) supports this idea. However, both calcifying groups (invertebrates and vertebrates) use proteins with similar biochemical properties, which suggests convergence in their biochemical strategies for calcification (Ettensohn, 2009).

On the other hand, several lines of evidence support the idea that some components of the calcification repertoire have an ancestral origin. Lowenstam and Margulis (1980) suggested that a tuning of ancestral intracellular calcium homeostasis and fluxes mechanisms that allowed the control deposition of calcium salts, preceded the initiation of calcification in the early Cambrian. For example, the enzyme carbonic anhydrase which ancestral function is to that catalyze the reversible conversion of

carbon dioxide into bicarbonate (one of the precursor ions for calcification) has been linked to calcification in a wide range of organisms such as sponges (Jackson *et al.,* 2007), corals (Isa and Yamazato, 1984) and vertebrates (Rajachar *et al.,* 2009).

One intriguing hypothesis suggests that calcifying matrices originated from changes in pre-existing ancestral epithelial mucus secretions (Marin *et al.*, 1996). The central idea is that non-calcifying precursors, for example mucins of the secretory system, gave rise to proteins able to regulate calcification. Mucins are heavily glycosylated and sulphated proteins that have characteristic tandem repeat organization able to form disulfide bonds through cross-linking of cysteine (Cys) enriched domains (Marin *et al.* 2000). In an attempt to test this hypothesis, Puverel *et al.,* (2005) used antibodies against the coral organic matrix in immunocytochemical analysis, but these did not label mucus-producing cells. Circumstantial evidence does, however, lend some support to the Marin hypothesis; proteins with mucin-like characteristics have been identified in calcifying and non-calcifying matrices from a diverse range of invertebrates (Fukuda *et al.* 2003; Marin *et al.* 2000).

Another piece of evidence that supports the idea of an ancestral calcification "tool kit" is the regulatory role of growth factors such as BMP-2 in mollusc shell formation (Matsushiro and Miyashita, 2004) and vertebrate ossification (Wonzney *et al.,* 1988). Furthermore the finding of a BMP-2/4 ortholog in corals (Hayward *et al.,* 2002) and its localization in the calcifying epithelium (Zoccola *et al.,* 2009) suggests an ancestral function for this family of proteins in calcification. Together these observations imply that extracellular matrix proteins may have been recruited on multiple occasions to roles in controlling calcification, as have carbonic anhydrases; calcification has evolved on multiple occasions but has been constrained in its choice of starting materials, blurring the distinction between conservation and convergence.

1. 2. Coral calcification.

The exoskeleton of scleractinian corals has been a particularly successful evolutionary innovation, and constitutes the framework of tropical reef ecosystems (Veron, 2000; Gunderson, 2007). Scleractinian corals originated and played a major reef-building role during the Triassic period (i.e. 248 mya) (Veron, 2000). This initial event in the

evolution of coral reefs was followed by periods of low abundance of scleractinian skeletons in the fossil record. Modern reefs have been dominated by scleractinians since the early Oligocene (33.7 mya) (Grigg, 1988).

Coral reefs contribute about 0.05% of net carbon dioxide (CO_2) fixation in the global oceans (Crossland *et al.* 1991). They are the most extensive biological structures on the planet, covering between 280-600 thousand square kilometers of the Earth's surface (Veron, 2000). This marine ecosystem, considered one of the most productive (Gunderson, 2007), is built upon a framework of scleractinian exoskeletons, which support a diverse community of organisms (Hatcher, 1997). Although coral reefs represent the net accumulation of calcium carbonate ($CaCO_3$) by both coral colonies and coralline algae (Kleypas *et al.,* 1999), scleractinian polyps are responsible for much of this $CaCO_3$ deposition. Scleractinian $CaCO_3$ deposition is estimated to be approximately 10 kg of $CaCO_3$ m^2/year, representing approximately half of the world's $CaCO_3$ production (Chave *et al.* 1975).

This elevated calcification rate is, in great part, the consequence of a symbiotic relationship between scleractinian corals and photosynthetic dinoflagellate algae from the genus *Symbiodinium* (Veron, 2000, Stanley, 2006). The algae use CO_2 and nitrogenous waste originated from coral metabolism while supplementing 90% of the host nutrition by translocating glycerol, sugars and amino acids (reviewed in Trench 1979). This symbiosis restricts coral reef distribution to shallow tropical waters due to the requirements for light availability for photosynthesis (Stanley, 2006; Veron, 2000) and to tropical and subtropical waters where temperature would allow the high calcification rates necessary for reef formation (Standley, 2006).

Coral reefs worldwide are in decline and are predicted to face even greater risks in the future due to decreased calcification rates as a consequence of ocean acidification (Kleypas *et al.* 1999; Hoegh-Guldberg *et al.,* 2007). Thus, a more detailed description of the cellular basis of coral calcification is urgently needed in order to more accurately predict and manage the future of coral reefs. Recent observations (Kleypas *et al.* 1999; Hoegh-Guldberg *et al.,* 2007), in combination with palaentological data (Lowenstein *et al.,* 2003; Ries *et al.* 2006), suggests that current trends on climate change will likely affect the distribution of coral reefs and their composition. A better

understanding of the coral biomineralization process is not only timely but also feasible given new molecular and genomic approaches being introduced into coral reef research.

In order to understand the mechanisms behind coral calcification, it is essential to examine the cellular biology of the calcifying cell (i.e. the calicoblast), including the genetic pathways that lead to: 1) calicoblast differentiation, and 2) calicoblast interactions with neighboring cell populations involved in ion transport and production of organic matrix precursors. So far there have been few attempts to examine the genetic pathways involved in calicoblast differentiation and/or secretion of organic matrix precursors during skeletogenesis in corals.

1. 3. Coral metamorphosis: the calicoblastic ectoderm.

Scleractinian corals are the only anthozoan group in which settlement and tissue reorganization during metamorphosis correlates with the deposition of a juvenile skeleton (Le Tessier, 1998; Clode and Marshall, 2004). During metamorphosis, coral planulae go through an oral endodermal tissue rearrangement that gives rise to the mesenteries and more extensive changes in the aboral ectoderm that give rise to the calcifying tissue; while the oral ectoderm retains the columnar organization seen in the planula, the aboral ectoderm is transformed from a columnar epithelium into a squamous calcifying cell type known as the "calicoblast" (Vandermeulen, 1975; Clode and Marshall 2004).

This newly formed epithelium secretes a macromolecular network of proteins, lipids and polysaccharides known as the organic matrix (OM) which controls mineral deposition by organizing $CaCO_3$ nucleation and determining the type of polymorph, as well as crystal size and shape (Fukuda et al. 2003; Clode and Marshall, 2003; Watanabe et al. 2003). Only calicoblastic cells in direct contact with the skeleton appear to be responsible for the synthesis and secretion of OM components (Puverel et al. 2005).

After settlement, the coral polyp secretes an early exoskeleton that is the precursor to adult colony morphology. The early skeleton differs from adult skeletons in both

structure and crystal type. While the early skeleton is lightly mineralised with smaller randomly oriented crystals of both calcite and aragonite (Vandermeulen and Watabe, 1973; Le Tessier, 1988), adult skeletons display a fibrous parallel growth that allows massive deposition of aragonite calcification (Vandermeulen and Watabe, 1973; Cuif and Dauphin, 2005).

In the coral *Pocillopora damicornis,* the basal plate and protosepta are the first skeletal elements to be formed (Vandermeuden and Watabe 1973). The basal plate has horizontally oriented crystals that are not present in adult skeletons, and its deposition involves the formation of spherulitic crystals that will subsequently fuse together (Vandermeuden, 1975). In *P. damicornis,* the parallel growth characteristic of adult calcification is evident 72 hours after settlement, co-incident with formation of the mesenteries (Vandermeuden and Watanabe, 1973).

Thus it is reasonable to think that: 1) early skeleton deposition is correlated with the aboral ectodermal transition from columnar cells to calicoblasts (Vandermeulen and Watabe, 1973; Le Tissier, 1988) and 2) while the base of the polyp is likely to be responsible for the first calcite- and aragonite-containing skeletal elements (i.e. basal septa & protosepta) the mesenteries may be responsible for the fiber-like calcification observed in coral colonies.

1. 4. The organic matrix: biological control over mineral deposition.

Organisms exert great control over mineral deposition for support structures. This biological control is reflected by the diversity of morphologies observed in calcified structures such as the variety of coral skeletons as well as in the different $CaCO_3$ polymorphs deposited by calcifying organisms. Scleractinian exoskeletons are composed predominantly of aragonite, in contrast to the spicules of alcyonarian soft corals, which are mostly composed of calcite (Rahman *et al.* 2006). In other well-studied groups there are also variations in $CaCO_3$ polymorph composition. For example, mollusc shells are most commonly composed of aragonite, but in some cases calcite predominates, and in other groups both calcite and aragonite crystals are present (Adaddi *et al.* 2006).

In scleractinian corals, as in other biomineralizing organisms, the OM is composed of proteins, lipids and polysaccharides (Cuif and Dauphin, 2005; Puverel *et al.* 2005a) and it has been shown that matrix biosynthesis, rather than calcium deposition may be the limiting factor controlling coral skeletogenesis (Allemand *et al.,* 1998). OM components are secreted to the extracellular space by the calcifying cells prior to mineralization (Goldberg 2001, Clode et Marshall 2002); this diverse network of organic molecules (Cuif and Dauphin, 2005; Puverel *et al.* 2005b) drives species-specific regulation of the biomineralization process. Each of the OM components fulfils a specific role such as control of crystal growth or construction of the matrix framework (Watanabe *et al.* 2003; Addadi *et al.* 2006; Nudelman *et al.* 2006). The presence of two types of vesicles in calicoblastic cells from the coral *G. fascicularis* suggests that OM precursors might be concentrated and transported in vesicles before being discharged to the mineralization site (Clode and Marshall, 2002b).

Data on scleractinian OM proteins is rather limited since only one protein, known as Galaxin, has been well characterized (Fukuda *et al.,* 2003). Galaxin was isolated from the OM of *Galaxea fascicularis* (Fukuda *et al.* 2003), and contains a Cys rich region arranged in tandem repeats with conserved di-Cys motifs, two putative N-Glycosylation sites and a N-terminal signal peptide sequence. The presence of these motifs has led some authors to suggest that the role of this protein in the OM is to form a macromolecular framework (Fukuda *et al.* 2003, Watanabe *et al.* 2003) as glycoproteins arranged in tandem repeat with conserved Cys residues are commonly found in many types of both extracellular matrix and cell surface receptor proteins (Gotliv *et al.* 2005; Bhattacharya *et al.* 2002; Pearsall *et al.* 2002). It is thought that tandem repeat structures allow the repeats to interact with each other or with other OM macromolecules (Gotliv *et al.* 2005; Sarashina & Endo, 2001).

Acidic calcium-binding proteins rich in Asp residues have been identified in both molluscs (Gotliv *et al.* 2005) and corals (Puverel *et al.* 2005), and have been implicated in calcification in both groups. Their calcium-binding activity, which is to date the only type observed in invertebrate matrix proteins, is related to the negatively charged carboxyl groups on Asp or Glu residues (Puverel *et al.* 2005; Rahman *et al.* 2006a). Furthermore, acidic proteins have been reported to control mineralization by interacting with calcium salts (Fu *et al.* 2005), and Poly-L aspartate molecules are

known to promote the precipitation of calcite rather that aragonite in alcyonarians (Rahman *et al.* 2006b). In the calcification literature, acidic matrix proteins are often associated with calcite deposition, although in some mollusc acidic proteins are able to nucleate aragonite (Nudelman *et al.* 2006).

Post-translational modifications of structural matrix proteins could arguably be a key factor determining coral colony morphology. Biosynthesis of N-linked glycoproteins appears to be a prerequisite for skeleton formation as reduced calcification rates were observed after inhibition of N-glycosylation (Allemand *et al.,* 1998). Proteins that are highly phosphorylated are thought to be involved in the inhibition of $CaCO_3$ nucleation due to their ability to interact with either calcium ions or $CaCO_3$ crystals as, for example, in the case of the crayfish protein, CAP-1 (Inoue *et al.* 2001). This is consistent with the idea that inorganic molecules, particularly phosphate and its derivates, are strong inhibitors of calcification (Simkiss, 1964).

Acidic polysaccharides have also been reported as major components of the OM in several marine invertebrates. Sulphated and acidic polysaccharides have been reported from the matrices of both scleractinians (Watanabe *et al.* 2003; Puverel *et al.* 2005) and alcyonarians (Cuif and Dauphin, 2005). Due to their dense negative charges, roles have been suggested for these molecules in both calcium transport during calcification and in positioning the matrix for the attachment of calcium binding proteins (Goldberg, 2001). In a similar way, it has also been suggested that fibrillar structures in the OM associated with sulphur may come from calcium binding polysaccharides and glycoproteins (Clode and Marshall, 2003). A core of carboxylate groups from Asp rich proteins surrounded by a ring of sulphate groups has been reported in the nacre nucleation sites of the cephalopod *Nautilus* (Adaddi *et al.* 2006; Nudelman *et al.* 2006). The putative function of the sulphate groups is to attract calcium ions to the calcification zones (Adaddi *et al.* 2006; Nudelman *et al.* 2006).

Different types of N-glycoproteins, as well as O-glycoproteins, have been in the control of calcification in the sea urchin OM (Ameye *et al.* 2001). N-glycoproteins are thought to be localized in amorphous subregions of the skeleton, whereas some O-glycoproteins are localized in subregions were skeletal growth is inhibited. O-glycoproteins contain sialic acid, which is known to interact with calcium. These

observations suggest that nucleation of $CaCO_3$ crystals during coral calcification is may also be regulated via glycosylation and phosphorylation of structural matrix proteins, which is consistent with Adaddi's (2006) idea that for controlled nucleation to occur, the OM must contain nucleation sites that will induce crystal formation more effectively than other charged locations. Despite this, the role of specific anion-cation interactions in calcification is not well understood.

1. 5. Calcium and carbonate: Sources and transport mechanisms.

Scleractinian polyps must exert control over the sources of ions in order to store and direct ions to the mineralization site to allow for $CaCO_3$ precipitation and crystallization. To achieve this, calcium and carbonate ions must be concentrated and isolated from the cellular environment. Two hypotheses have been postulated regarding calcium transport mechanisms to the calcification site: 1) calcium transport occurs via an active transcellular route across the oral epithelium to the calcification site (Tambutté *et al.,* 1996; Clode and Marshall, 2002b) and/or 2) calcium reaches the mineralization site via a paracellular route by passive diffusion of calcium ions (Tambutté *et al.,* 1995).

According to Clode and Marshall, (2002b), oral ectodermal mucus secretions may facilitate intracellular calcium uptake by oral cell population. This might be achieved by allowing higher calcium ion concentrations within the mucus matrix than in the surrounding seawater. The observation that, in *A. millepora* embryos, the oral pore differs from the surrounding ectodermal tissue and is composed almost exclusively of secretary cells (Ball *et al.* 2002) is consistent with this idea. However, concentration of calcium ions by oral mucus secretions is as yet unproven, and the fact that the concentration of calcium in seawater is already 10^5 fold higher that that of cytoplasm suggests that mucus is unlikely to play an important role in calcium concentration (Allemand, per. comm.).

Tambutté *et al.,* (1996) have demonstrated that calcium uptake by coral tissue is a prerequisite for skeletogenesis, and suggest a transcellular route involving a L-type voltage-dependent Ca^{2+} channel (Zoccola et al. 1999) and a plasma-membrane calcium pump (PMCA) (Zoccola et al. 2004). Under this model, transport of calcium

across calicoblastic cells is mediated by calcium-binding proteins and is dependent on the cytoskeleton. Intracellular vesicles identified in the calicoblastic ectoderm of a variety of scleractinian corals may be involved in packaging of calcium and organic matrix precursors prior secretion to the calcification site (Clode and Marshall, 2002).

1. 6. Carbonate ions: The divalent anion.

Whilst the only available source of the calcium ions required for skeleton deposition is the external medium, inorganic carbonate could theoretically be acquired either from the surrounding seawater (as bicarbonate or carbonate) or from coral respiration (in the form of CO_2) (Allemand *et al.* 2004). Erez (1978) and Furla *et al.* (2000) have shown that metabolic CO_2 is the major source of carbon for calcification. The finding of high numbers of mitochondria in calicoblastic cells (Clode and Marshall, 2002; Allemand *et al.* 2004) is consistent with this idea. CO_2 can diffuse across cell membranes passively, whereas HCO^-_3 and CO_3^{2-} require specific protein carriers to do so (Allemand *et al.* 2004).

The enzyme carbonic anhydrase (CA) catalyses the reversible interconversion of CO_2 and HCO^-_3, the latter being one of the substrates necessary for $CaCO_3$ deposition (Tambutté *et al.* 1996; Allemand *et al.* 2004). CA has been demonstrated in the skeletogenic cell layer of the azooxanthellate coral *Tubastrea aurea* (Tambutté et al. 2007) and in the calicoblastic ectoderm of the zooxanthellate coral *Stylophora pistillata* (Moya et al, 2008). Furthermore, CA activity has been reported in the calicoblastic ectoderm of *Acropora hebes* (Isa and Yamazato, 1984) and a role for CA in skeletogenesis has been demonstrated in the coralline demosponge *Astrosclera willeyana* (Jackson *et al.* 2007).

Moya *et al.,* (2008) have proposed that CO_2 diffusing out of the calicoblastic cells as a consequence of the high pH of this calcifying region (Al-Horani *et al.,* 2003) may be converted into HCO_3 by the secreted CA. The protons produced at the site of calcification could be removed by the Ca-ATPase present within the calicoblastic epithelium (Zoccola *et al,* 2004). In addition to the passive movement of CO_2 it has also been proposed that HCO_3^- is transported by the calicoblastic cells to the calcifying site (Furla *et al.,* 2000). Given that mammalian HCO_3^-/Cl^- exchangers are

known whose role is to compensate for increases in intracellular pH by exporting HCO^-_3 ions (Phillips *et al.* 2002), intracellular pH regulation mechanisms might also have roles in HCO^-_3 transport to the calcification site. However, very little is known about cellular pH regulation in cnidarians and more research is nessesary to understand whether metabolic CO_2 rather that HCO_3^- is the main carbon source during calcification.

1. 7. Calcium signaling: Calcium mediated calcium carbonate deposition.

Calcium is the most versatile and widely used secondary messenger in eukaryotes; some of its signaling mechanisms are conserved across phylogenetically distant groups (Haeseleer *et al.* 2002, Hofer and Brown, 2003). Due to its particular flexibility as a ligand, changes in intracellular calcium concentration regulate a variety of cellular processes ranging from fertilization to apoptosis (Carafoli, 2005; Parekh, 2006), and these processes generally involve calcium sensor proteins, the best known of which is calmodulin. Since calcification is a process involving ion channel regulation, vesicle trafficking and the synthesis of OM precursors, it is likely that calcium sensor proteins and their signaling pathways play important roles in coral skeletogenesis.

Calcium dynamics, homeostasis and signaling are regulated by calcium itself via calcium binding proteins known as calcium sensors. These proteins are able to bind calcium depending on the intracellular calcium concentration. As a result of this interaction, these molecules experience topological changes that allow them to interact with target proteins located downstream in the signaling cascade (Burgoyne and Weiss. 2001). Eukaryotic cells require calcium in the cytosol as a second messenger to regulate a large number of processes. To enable these processes, cells must maintain a low intracellular concentration (~ 0.1 μM) so that elevations in concentration (10-20 fold) can be used to regulate cellular functions (Lee and Klevit, 2000), thus avoiding abnormal increases in cytosolic calcium concentration, which may lead to cell death (Carafoli, 2003). These observations reveal a key role of calcium as a potent activator of gene transcription (Berridge *et al.* 2003) and suggest an important role for this secondary messenger during coral calcification.

It appears that calcium ion uptake/exit by the calicoblastic ectoderm in *Stylophora pistillata* is facilitated by Ca^{2+} channels (Zoccola *et al.,* 1999) and Ca^{2+} ATPases (Zoccola *et al.,* 2004) respectively. According to (Zoccola *et al.,* 1999), scleractinian corals have two distinct cell types with respect to sensitivity to Ca^{2+} channel inhibitors. The first type includes calcium-transporting cells that belong to the calicoblastic epithelium and contain L-type voltage dependent Ca^{2+} channels. The second type includes all other cells that do not transport calcium actively and lack L-type voltage dependent Ca^{2+} channels (Zoccola *et al.* 1999). Despite this, it remains unclear whether calcium reach the calcification site as ions or insolubilized by attaching to OM precursors such as lipids or proteins. However, the fact that L-type voltage dependent Ca^{2+} channels are present in calicoblastic cells suggests that this cell population is actively involved in sequestering calcium during skeletogenesis.

The calcium channels identified in cnidarians (jellyfish and coral; Zoccola *et al.* 1999; Jeziorsky *et al.* 1998) are members of the metazoan L-type family of channels. The $\alpha 1$ subunit is an integral membrane protein, which directs the permeation of calcium in a voltage-dependent manner (Walker and Waard, 1998 and Jeziorsky *et al.* 2000). This protein has been isolated along with a calcium ATPase from the coral *S. pistillata* that localizes to the calicoblast ectoderm (Zoccola *et al.* 2004).

Studies of calcium signaling in cnidarians have focused specifically on calcium channels (Zoccola *et al.,* 1999; Jeziorsky *et al.* 1998) and ATPases (Zoccola *et al.,* 2004), one of the few exceptions to this being the identification of a novel calcium sensor in the sea anemone *Anthopleura elegantissima* (Hauck *et al.,* 2007). Very little is known about calcium transduction mechanisms in cnidarians or their calcium sensor complements (Haeseleer *et al.,* 2002). Given their likely significance in regulation of processes such as metamorphosis and calcification, a survey of calcium sensor proteins is likely to yield insights into many aspects of coral biology.

1. 8. Calcification and photosynthesis: Light-enhanced calcification.

High rates of calcium carbonate deposition in the oceans are generally associated with high rates of photosynthesis (Gattuso *et al.*, 1995). This link is established via

symbiotic relationships between photosynthetic algae and organisms such as foraminiferans (reviewed in Lee *et al*., 1979), giant clams (Fitt *et al.*, 1986) and scleractinian corals (Goreau, 1959). Scleractinian calcification accounts for most of the $CaCO_3$ precipitation on tropical reefs. This rapid growth and calcification rate in a nutrient-deficient environment can be explained by the photosynthetic activity of dinoflagellates from the genus *Symbiodinium* (Stanley, 2006).

It has been known for some time that photosynthesis enhances the ability of a coral to calcify (Goreau, 1959; Chalker and Taylor 1975). Le Tissier (1988) reported that whereas adult skeleton formation normally follows a diurnal cycle, under constant light conditions, light-enhanced calcification appears to be dependent largely on the light regime (Le Tissier 1988, Moya *et al.,* 2006).

In *Stylophora pistillata* the production of metabolic CO_2 is at least six times higher during the day than is required for calcification (Furla *et al.,* 2000). The use of metabolic CO_2 requires carbonic anhydrase (CA) activity. Moya *et al.,* (2008) have shown that the expression of CA is twice higher in the dark than in the light and suggest that a secreated CA may then help to sustain the CO_2 hydration rate in the dark where the sub-calicoblastic medium is more acidic than in the light. However since CA expression is enhanced in the dark and not in the light, it is likely not to be directly involved in light-enhanced calcification (LEC).

One role for zooxanthellae in the LEC process may be secretion of OH^- during photosynthesis, which could buffer the protons produced during calcification (Allemand *et al.,* 1998). Indeed the kinetics of OH^- production during light/dark and dark/light transitions (Furla *et al.,* 2000) are consistent with the lag-phase necessary to switch between light/dark and dark/light values of calcification (Moya *et al.,* 2006). The kinetics for OM synthesis between dark and light conditions (Allemand *et al.,* 1998) are also consistent with the lag-phase necessary to switch between dark and light calcification values (Moya *et al.,* 2006).

The interaction between photosynthesis and calcification is not simple; not only is there photosynthesis-enhanced $CaCO_3$ deposition, but also there are suggestions that calcification might enhance photosynthesis (Mcconnaughey, 1994). Hence the

cellular mechanisms regulating coral calcification must be correspondingly complex. Respiration, calcification and photosynthesis are processes that must be tightly linked in symbiotic scleractinian corals, and disentangling the interactions between these cellular processes is key to understanding skeletogenesis in reef building corals.

This thesis focuses on late developmental stages in the reef-building staghorn coral *Acropora millepora,* which span metamorphosis, settlement and the initiation of skeletogenesis. Early calcification in *A. millepora* represents an ideal system to study the cellular mechanisms underlying skeleton deposition in scleractinian corals as it is not enhanced or modified by *Symbiodinium sp* photosynthesis (due to the aposymbiotic nature of *A. millepora* larvae) while providing direct access to the calicoblastic ectoderm without the complications associated to a massive adult skeleton.

Using an ongoing EST project on different developmental stages of *A. millepora*, we focused on identifying and characterising both calcifying matrix components from the Galaxin family as well as calcium sensors proteins potentially able to regulate and translate the calcium signal during settlement and metamorphosis. Three Galaxin related genes were identified, whose expression patterns overlap during development and are consistent with roles in early and adult calcification (Chapter 3). In a similar way, a novel and coral-specific calcium sensor protein was identified that interacts with calmodulin in vitro and is expressed during settlement and early stages of skeleton deposition (Chapter 4).

In parallel, primary cell cultures were established from five key developmental stages; the technique reported here will allow the isolation of embryonic coral cell populations and should thus facilitate the dissection of cell differentiation pathways and cell lineages in corals (Chapter 2). Finally, microarray analysis was used to investigate changes in gene expression during the early post-settlement phase during which calcification is initiated (Chapter 5).

Although the results reported here are preliminary and reveal a high level of complexity, hopefully they will facilitate the elucidation of the gene networks involved in processes such as lineage specific cell differentiation, the initiation of

skeleton deposition and the establishment of symbiosis. In particular, the expression data reported for 1189 differentially expressed genes through the early post-settlement time window should provide a valuable resource for understanding the calicoblast differentiation and early skeletogenesis in the scleractinian coral *A. millepora*.

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CHAPTER 2. In vitro culture of cells derived from larvae of the staghorn coral *Acropora millepora.*

2.1. ABSTRACT.

Previous attempts to culture cells from corals or other cnidarians have been unsuccessful. These efforts have, however, generally made use of adult tissue as starting material. Early developmental stages are potentially more appropriate for the initiation of cell cultures, as the expectation is that a greater proportion of the cell population is undifferentiated and may have the intrinsic ability of unlimited cell renewal. To explore this idea, cell cultures were initiated from five key stages of coral development, and the presence of coral cells monitored by polymerase chain reaction (PCR) using coral-specific primers. After four weeks, semi-quantitative PCR implied that coral cells were better represented in cultures initiated from planulae than in those derived from earlier developmental stages. Coral cells were detected in cultures initiated from planulae for up to ten weeks, but after this time extensive contamination by the protist *Thraustochytrium* sp was observed.

2. 2. INTRODUCTION.

Despite the ecological importance of corals and widespread concern regarding the threats posed to coral reefs by global climate change, little is known about the molecular bases of many aspects of their biology such as metamorphosis, symbiosis and calcification. The size and complexity of coral colonies complicate empirical analyses, and cell lines derived from corals would greatly facilitate molecular analyses of many aspects of their biology. A few studies have focused on the use of primary cultures of differentiated cells derived from adult colonies to perform short-term assays (Domart-Coulon *et al.* 2001*,* 2004; Helman *et al,* 2008). Previous attempts to establish stable coral cell lines have been hampered by contamination or short-term survival of isolated cells and low cell division rates (Frank *et al.* 1994; Kopecky and Ostrander 1999).

In these previous studies, coral cells were identified and characterised on the basis of morphology, but the appearance of cells *in vitro* does not necessarily correspond to

that *in vivo* (Frank *et al.* 1994). Molecular markers can potentially assess the nature of cultured cells as well as discriminate between different cell types and some preliminary attempts have been made to apply these approaches. Puverel *et al.* (2005) developed antibodies against the organic matrix of *Stylophora pistillata* and used these to identify putative calicoblast cells in culture and Helman *et al.* (2008) used universal 18S rDNA primers to assess the nature of cultured cells employed for the study of the production of coral extracellular matrices *in vitro*.

In this study, coral specific markers were employed to monitor the presence of coral cells in primary cultures derived from a variety of embryonic and larval developmental stages. The results imply that an embryonic cell type present in planulae may be the most appropriate starting material for cell cultures. The methods described should greatly facilitate the dissection of cell differentiation pathways in corals.

2. 3. MATERIAL AND METHODS.

2. 3. 1. Collection of embryos.

Acropora millepora mature colonies were collected during the 2005-spawning event at Magnetic Island (Lat: 19° 7' 60 S, Long: 146° 49' 60 E, Queensland, Australia). Following spawning, gametes from several colonies were mixed to facilitate fertilisation, and the resulting embryos maintained in containers containing fresh seawater for 2h. At 15 hours post-fertilization, embryos selected for cell culture were transferred to 190mm petri dishes containing 0.2μm filtered seawater (FSW) at densities no higher than 600 embryos per dish. They were washed 5 times with 0.2μm FSW. Water was changed 2 times per day. Developing embryos selected for cell cultures were maintained at 23°C and on a 12-hour light/12-hour dark cycle.

2. 3. 2. Cell culture.

Prior to initiation of cell cultures, individual coral embryos/larvae (50 per each stage) were treated for 15 minutes in 5 ml aliquots of sterile seawater containing ampicillin (270μg/ml), streptomycin (135μg/ml), and chloramphenicol (27 μg/ml). This process

was repeated three times. Single embryos/larvae were transferred to 5 ml of cell culture medium (10% DMEM GIBCO, 5%FBS GIBCO, 3% Antibiotics/antimycotics SIGMA, 2% marine enrichment F/2 SIGMA and 80% 0.2μm FSW) in individual wells on a 6 well culture plate, and incubated at 23°C in the dark. Coral embryos/larvae dissociated spontaneously after the addition of antibiotics/antimycotics (Fig 1A). After a week of incubation, embryos / larvae were totally dissociated irrespective of developmental stage of the starting material, and individual cells were observed in each well. The nature of the cells in culture was monitored every two weeks, and subculturing carried out when cells approached confluence.

Subculturing was achieved by harvesting the cells from 5ml cultures by centrifugation at 1200 rpm followed by resuspension of the resulting cell pellets in 2 ml aliquots of fresh medium. Pellets were washed 3X with 2 ml aliquots of fresh media. 0.5 ml volumes of the ultimate suspension were used to inoculate 4.5 ml aliquots of fresh media in a new cell culture plate. In each case the residual volume (1.5 ml) was used for DNA extraction.

2. 3. 3. Monitoring the nature of cells in culture.

Total DNA was extracted (DNeasy Tissue; QIAGEN) from cultures, and used as template in PCR using the primers described in Table 1. The universal primer pair (nuclear ribosomal small subunit – universal18s) was used to detect contaminating eukaryotic organisms. The remaining primers were coral-specific, and amplify either a 500bp-600bp fragment from the Pax-C intron (van Oppen *et al.* 2001) or a 250bp fragment from an exon of a Red Fluorescent Protein (RFP). PCR products were subjected to DNA sequencing using the amplification primers to enable identification of contaminating organisms and/or verify the presence of *Acropora* cells in culture.

2. 3. 4. Polymerase chain reaction (PCR).

PCR reactions contained in a total volume of 20 μl : 1μl of primers (10mM), 2.5 μl dNTP's (2mM each), 2μl $MgCl_2$ (25mM), 2μl 10x Taq Buffer, and 0.1-0.5 Units of

Taq polymerase. Cycling-conditions involved denaturing and annealing times of 30 seconds and extension time of 60 seconds, followed by a final extended extension incubation time of 2 minutes. Generally 30 cycles was sufficient for the amplification DNA. Annealing temperatures used were 3 to 5°C below the melting temperature of the primers involved (Table 1). PCR products were run on 1% agarose gels and amplified bands recovered (Gel extraction Kit QIAGEN) for sequencing. Genomic DNA extracted from sperm derived from spawning *A, millepora* colonies was used for positive controls.

2. 3. 5. DNA sequencing.

Template for DNA sequencing was prepared in a thermocycler using the ET Dye Terminator Sequencing Kit from Amersham Biosciences. The cycle sequencing protocol for the DYEnamic™ ET Dye kit used was: 95°C for 20 seconds, 50°C for 15 seconds, 60°C for 3 minutes (35 cycles). Reactions were analysed on a MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences) via a laser induced fluorescence capillary system. Sequences were manually checked and assembled with Sequencher Version 3.1or 4.0.

2. 3. 6. Microscopy.

Differential interference contrast (DIC) microscopy was carried out using an Olympus BX51 microscope fitted with a CCD camera Olympus DP70.

2. 4. RESULTS AND DISCUSSION.

2. 4. 1. Cell cultures originated from planula tissues gave higher PCR yields than those started from early stages.

Movement and migration of specific cell populations during gastrulation gives rise to the two germ layers present in cnidarians (i.e. ectoderm and endoderm) and a variety of tissue specific cell types (Hyman 1940; Ball *et al.* 2002). In hydrozoans, an interstitial population of undifferentiated cells (I-cells) in a constant mitotic cycle is

also founded during gastrulation (Hyman 1940; Martin and Archer 1997; Bode 1996). This cell population migrates and differentiates into tissue specific cell types during metamorphosis, asexual reproduction and/or regenerative processes (Hyman 1940; Martin 1990; Bode 1996). The characteritistics of pluripotent cell types such as I-cells suggest that, under appropriate conditions, they may be capable of founding cell cultures.

To test the idea that a stem cell-like population is present throughout early coral development, cell cultures were initiated from five key developmental stages: 1) pre-gastrulation stage "prawnchip" (PC), 2) gastrulating embryo "donut" (D), 3) late gastrula "sphere" (S), 4) early planula "pear" (Per) and 5) planula larva (Pla).

After 4 weeks, cell cultures initiated from the late stages of coral development characteristically gave higher yields in semi-quantitative PCR with all 3 primer pairs than those started from early stages, with planula giving overall highest yields (Fig 1D-F). In most cases amplified products matched in size and sequence those obtained from a coral genomic DNA positive control (+), Pax-C intron (~500 bp product), RFP (~250 bp product) and coral 18S rDNA (~250 bp product). However, four-week cultures derived from the prawnchip stage material were acidic and yielded an 18S rDNA amplification product, which differed in size from that expected (Fig 1F) and was assumed to indicate contamination. At this time, cultures initiated from planula larvae were selected for longer-term analysis, and those initiated from other stages discarded.

Coral signal was detected (with each of the 3 primer sets) for approximately ten weeks in cultures initiated from planula tissue (1D-F). After 12 weeks, however, no coral specific PCR products could be obtained and only a ~2000bp product was amplified with the universal 18S rDNA primers (Fig 2 A-B). Comparison of the sequence of this product with the NCBI nucleotide database identified it as derived from *Thraustochytrium* sp. BS2 (86% identity, E-value = 0.0), a parasitic marine protist (Labyrinthulida: Stramenopiles) that is a recurrent contaminant of coral cell cultures (Rinkenvich 1999) (Fig 2C).

Microscopy conducted on prawnchip (Fig 3A-B) and post-gastrulation stage (Fig 3C-H) identified a small rounded cell type whose morphology resembles that of interstitial cells from hydrozoans (Hyman 1940). Cells with this same morphology were observed in primary cultures for the period during which coral cells were known to be present based on PCR analysis. These cells are assumed to be of coral origin and appeared to be actively dividing, forming aggregates that mainly remained in suspension and did not attach to the culture substrate (1B-C). Although no attempts were made to count the cell present, the fact that one round of subculturing every two weeks was necessary to reduce cell numbers implies that these cells were dividing.

Whilst cultures derived from post-gastrulation material were initially heterogenous (Fig 3C-H), after four weeks in culture the only cell type that could be recognised was a small rounded cell population that formed cell aggregates. We cannot discern whether this cell population corresponds directly to those cells with the same morphology in larval material. There is evidence that some cnidarian cells in culture are capable of transdifferentiation (Schmid and Reber-Muller 1995), potentially complicating comparison of *in vivo* and *in vitro* morphology. In the case of *Hydra*, stem cell markers have been developed (Mochizuki *et al.* 2000) and a similar approach could be useful in investigating the nature of the coral cells in culture.

The interstitial cell lineage of hydrozoans is relatively well characterised (Martin and Archer 1997; Bode 1996). Martin (1990) described a mechanism in the hydrozoan *Pennaria tiarella,* by which aboral interstitial endodermal cells initiated neuronal differentiation in the endoderm to then migrate to the ectoderm where differentiation is complete at the time of settlement and metamorphosis. This interstitial cell population increases in number during development, reaching its peak at the planula stage (Martin and Archer 1997).

Although the literature describing I-cells in anthozoans is limited and has focused mainly in gametes differentiation (Gaino *et al,* 2008), several lines of evidence imply that a corresponding pluripotent cell type is present. For example, without a stem cell-like precursor it is difficult to account for the adult specific cell types (i.e. calicoblasts), which appear only after metamorphosis. In addition, all of the cell types known to be derived from I-cells in hydrozoans (gland cells, neurons, nematocysts

and gametes; Bode 1996) are present in anthozoans (Hyman 1940). These results suggest the presence in coral larva of a cell population whose number increases during late development and which is capable of founding cell cultures.

The extent to which cellular differentiation mechanisms in corals resemble those described for hydrozoans and the role of stem cells as precursors of adult specific cell types remain unclear. In any case the ability to maintain cells derived from coral planulae in culture for long periods of time should facilitate the study of many diverse aspects of coral biology.

2. 4. 2. Thraustochytrids are recurrent contaminants of coral cell cultures.

Despite extensive washing of embryonic and larval coral material prior to the establishment of cell cultures, PCR analysis indicated that cultures initiated from prawnchip (PC) material had become contaminated. Although DNA sequence data were not obtained, this product at approximately ~ 700 bp was substantially different in size from that of the expected coral product i.e. ~ 250 bp (Fig 1F). It is likely that, as in the case of the 12 weeks planula-derived cultures, the contaminant is *Traustochytrium.* Thraustochytrid protists appear to be a persistent problem in establishing cell cultures from corals (Rinkevich, 1999) and other marine invertebrates (Frank *et al.,* 1994). Thraustochytrids have also been identified in epithelial mucus secretions from the scleractinian coral *Fungia granulosa.* It has been suggested that this group of protists may provide nutritional sources to the coral host (Harel *et al.,* 2008).

Due to both the diversity of marine protozoans present in seawater, and the flexibility of coral tissues to establish parasitic and/or symbiotic relationships with other marine unicellular organisms, the identification of cultured cells originated from coral tissues needs to be carefully assessed. Cell identity can be tested by PCR with coral specific markers as showed in this study and cultured cells could be sorted according to size, mitotic cycle or fluorescent probes using cell sorting (FACS) technology. The three-month period in which coral cells dominated the cultures provide a period in which short-term experiments can be performed and coral cell isolation achieved.

2. 5. CHAPTER FIGURES AND TABLES.

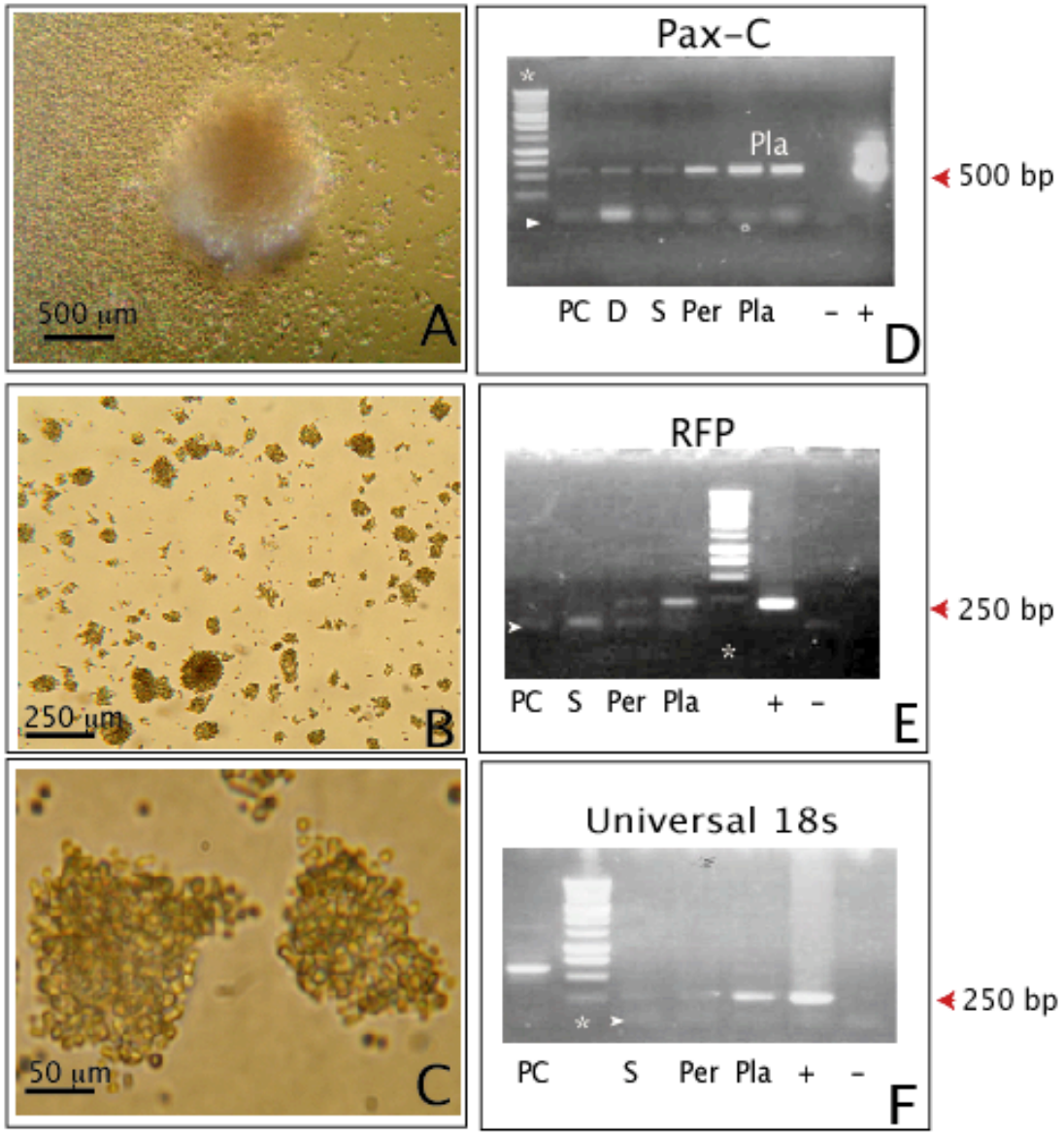

Figure 2. 1. Cell aggregates and PCR identification of cultured cells.
Coral embryos/larvae dissociated spontaneously after the addition of antibiotics. Single cultures were initiated from one embryo/larvae for each developmental stage (A). After a month, cultures formed cell aggregates that remained in suspension (B-C). After 4 weeks, cells cultures initiated from planula larvae (Pla) gave consistently higher PCR yields than did any other stage (D to F). Positive control (+). Negative control (-). Primer dimers (white arrow heads). DNA markers (asterisks). Prawnchip (PC), Donut (D), Sphere (S), Pear (Per), Planula (Pla).

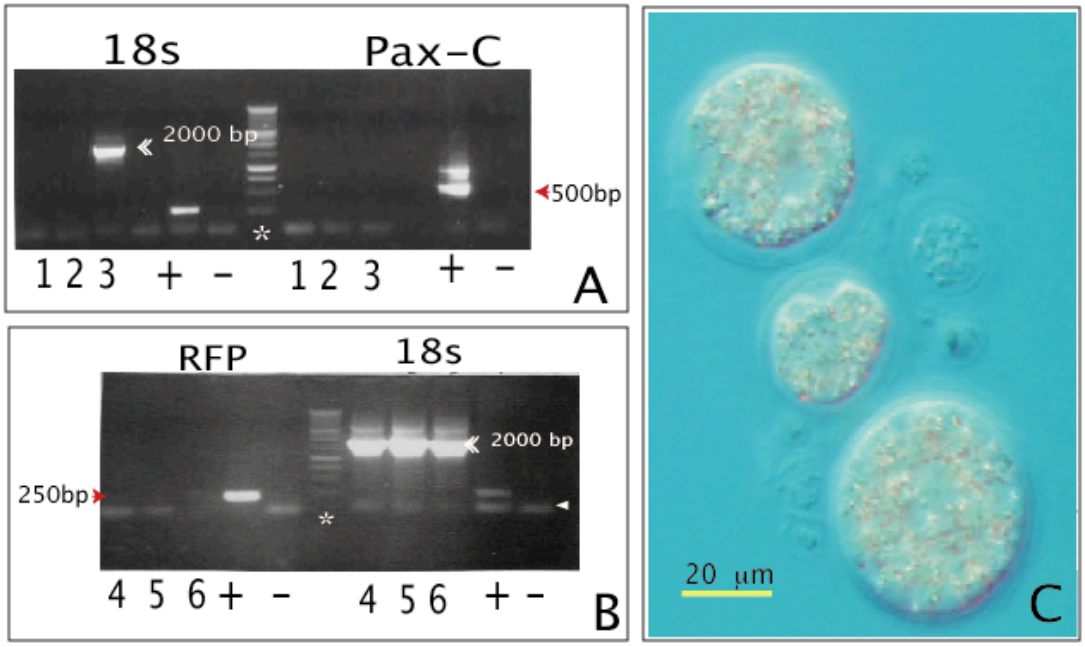

Figure 2. 2. Thraustochytrids overgrow coral cells in culture.
After 3 months of culture, coral-specific primers gave negative results, and a ~ 2000bp (double arrow) product was amplified with universal 18S rDNA primers (A and B). This band was identified as derived from *Thraustochytrium sp* (C). Numbers 1 to 6 represent single 3 month-old cultures originated from Planula larva. Positive control (+). Negative control (-). Primer dimers (white arrow heads). DNA markers (asterisks).

Table 2. 1. PCR markers for cell identification.

Marker	Direction	Sequence	Tm ^{o}C
Uni18s	Fwd:	5'CGAATTCAACCTGGTTGATCCTGCCAGT3'	61
	Rev:	5'CCGGATCCTGATCCTTCTGCAGGTTCACCTAC3'	65
Pax-C	Fwd:	5'TCCAGAGCAGTTAGAGATGCTGG3'	60
Intron	Rev:	5'GGCGATTTGAGAACCAAACCTGTA3"	56
RFP	Fwd:	5'GGAGAGCAGACAGAGAAGC3'	53
	Rev:	5'GGAATCATTGGTGACAGTACA3'.	50

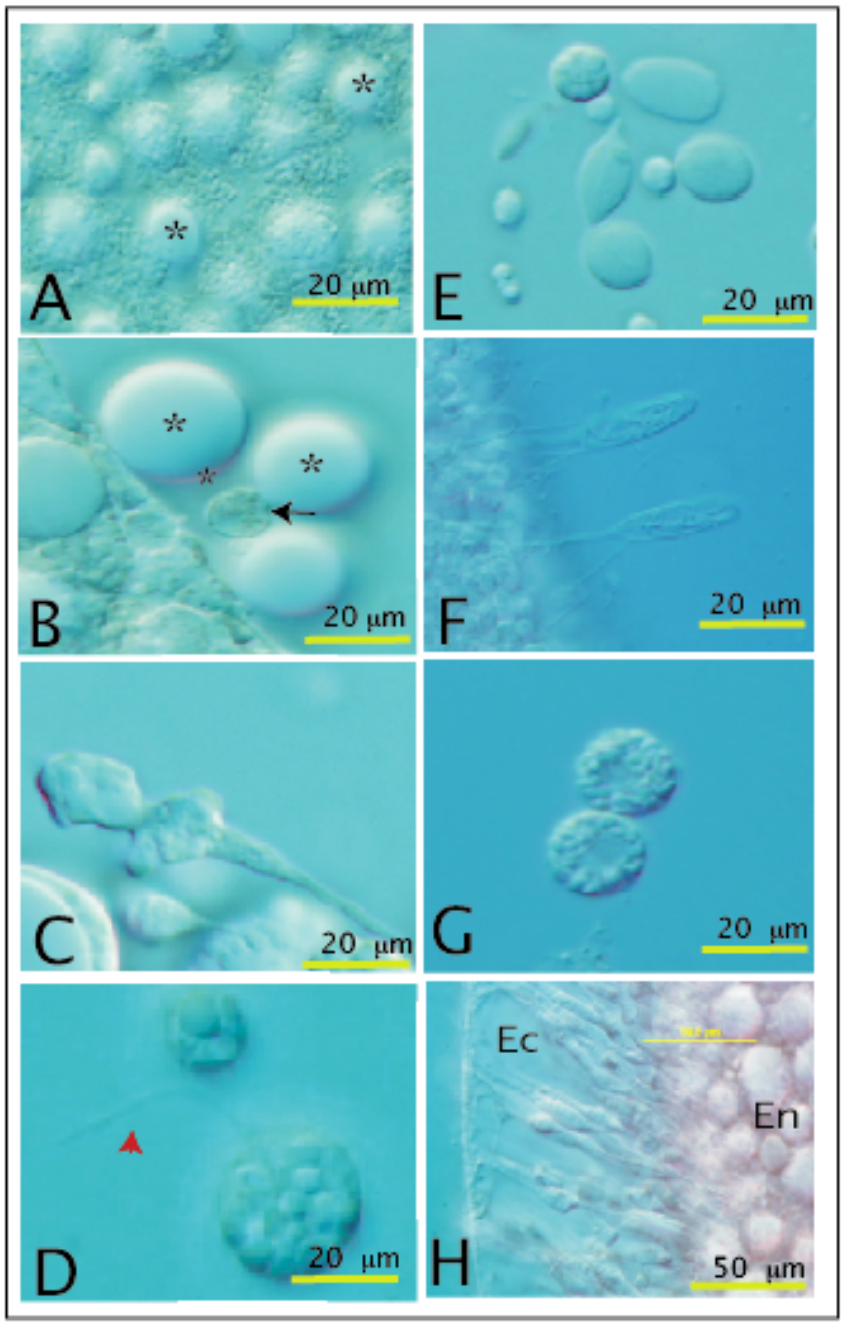

Figure 2. 3. Cell diversity during coral development.
Prior to gastrulation, coral embryos consist predominantly of a single morphological cell type (a-b) (black arrows) surrounding lipid-containing vesicles (asterisk). After gastrulation, a range of cell morphologies can be observed; these cell types can be identified at least until the planula stage (c-h). Elongated columnar cells (c), flagellated cells (d), nematocystes (e-f), and small round cells (g) were observed after establishment of the tissue layers (h). Endoderm (En), Ectoderm (Ec). Flagellum (red arrow).

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CHAPTER 3. Differential expression of three galaxin-related genes during settlement and metamorphosis in the scleractinian coral *Acropora millepora.*

3. 1. ABSTRACT.

The coral skeleton consists of $CaCO_3$ deposited upon an organic matrix primarily as aragonite. Currently galaxin, from *Galaxea fascicularis*, is the only soluble organic matrix component that has been characterized from a coral. Three genes related to *galaxin* were identified in the coral *Acropora millepora*. One of the *Acropora* genes (*Amgalaxin*) encodes a clear galaxin ortholog, while the others (*Amgalaxin-like 1* and *Amgalaxin-like 2*) encode larger and more divergent proteins. All three proteins are predicted to be extracellular and share common structural features, most notably the presence of repetitive motifs containing dicysteine residues. In situ hybridisation reveals distinct, but partially overlapping, spatial expression of the genes in patterns consistent with distinct roles in calcification. Both of the *Amgalaxin-like* genes are expressed exclusively in the early stages of calcification, while *Amgalaxin* continues to be expressed in the adult, consistent with the situation in the coral *Galaxea.*

3. 2. INTRODUCTION.

Although calcification – the deposition of calcium salts, usually to provide a form of skeletal support - is a widespread trait among animals, scleractinian corals are distinguished by the scale on which they carry out this process. In contrast to the internal skeletons of tetrapods and bony fish, which are based on calcium phosphate in the form of hydroxyapatite, many invertebrate skeletons are composed of $CaCO_3$. This may take the form of either calcite or aragonite, which have distinct structural properties. Both forms are known in cnidarians and molluscs, with the massive exoskeletons of extant scleractinian corals consisting predominantly of the latter.

Scleractinians are responsible for the underlying framework of coral reefs and are the most obvious calcifying animals in warm shallow waters of the tropics and sub-tropics. Calcification by corals is known to be strongly enhanced by light (e.g. Muscatine et al, 2005; Moya et al., 2006) and is therefore assumed to be driven

indirectly by photosynthesis in their dinoflagellate symbionts, but very little is known of the mechanism of calcification or the nature of the organic matrix on which the $CaCO_3$ of the skeleton is deposited.

The difference in the type of $CaCO_3$ polymorph used to build the wide range of invertebrate support structures reflects the biological control exerted by the organism during mineral deposition. This is achieved via a macromolecular network of proteins, lipids and polysaccharides (Cuif and Dauphin, 2005; Puverel et al., 2005, Helman et al., 2008) known as the skeletal organic matrix (SOM). Prior to mineralization, this matrix is secreted to the extracellular space by the calcifying cells and there induces $CaCO_3$ nucleation, determining the type of polymorph as well as size and shape of crystals (Allemand et al, 1998: Goldberg, 2001; Fukuda et al., 2003; Clode and Marshall, 2002a; Clode and Marshall, 2003; Watanabe *et al.,* 2003).

Although the process of calcification and the nature of the calcifying matrix have been extensively studied in molluscs (Adaddi et al*.,* 2006; Fu et al. 2005; Gotliv *et al.,* 2005), there have been relatively few corresponding studies on corals. To date, the only protein to have been fully characterised from the calcifying matrix of scleractinian corals is galaxin, which was originally identified from the coral *Galaxea fascicularis* (Fukuda *et al.,* 2003). Most studies carried out to date have focussed on soluble components of adult coral SOMs, despite the fact that calcification is initiated immediately after settlement (Vandermeuden, 1975).

The presence of the massive skeleton, however, complicates analysis of the process of calcification, whereas the period immediately following settlement is likely to be more tractable. The initiation of larval skeleton deposition correlates with the aboral ectodermal transition that gives rise to the calicoblastic ectoderm (Le Tessier, 1988; Vandermeulen, 1975; Clode and Marshall, 2004, Hirose et al., 2008). Larval skeleton deposition does not require the presence of zooxanthellae, but generates a template for the skeleton of the initial polyp (Vandermeulen, 1975; Le Tessier, 1988; Cuif and Dauphin, 2005). Both the organization and orientation of calcium carbonate differs between larval and adult calcification; larval skeletons are more lightly mineralised and may contain amorphous calcium carbonate, calcite and aragonite crystals.

When the latter are present they are in the form of smaller crystals that are randomly orientated whereas adult skeletons are exclusively composed of aragonite crystals organized in parallel fibres (Vandermeulen and Watabe, 1973). It is unclear whether this apparent difference between larval and adult calcification patterns represents two distinct types of calcification, or ontogenetic stages in the development of a largely aragonite skeleton as is the case in molluscs (Weiss et al, 2002). In either case, the immediate post-settlement period is the only time during the life cycle of the coral that calcification, and the role of the SOM can be studied in isolation and it thus presents a unique opportunity to investigate the molecular bases of coral calcification.

An ongoing EST project on the staghorn coral *Acropora millepora* has allowed the identification of genes encoding candidate calcifying matrix components. Here we report the sequences and expression patterns of three genes encoding galaxin-related molecules in *Acropora*. Based on these data, we suggest distinct roles for the three gene products during the two characteristic phases of calcification observed during the coral life cycle. This paper is particularly topical given the current concern over the effects of rising levels of atmospheric CO_2 on the acidity of the oceans, and consequently on the ability of marine organisms to lay down their $CaCO_3$ skeletons.

Note that some of the results presented in this chapter were obtained by others, but are included here to provide context for the work that I did. The *Galaxin-like2* gene was cloned by David Hayward, and the in situs presented were obtained by Zhiyi Lin and Eldon Ball, after extensive trouble shooting by the Ball laboratory. In situ hybridisation analysis of the galaxins was a particularly challenging undertaking, and required modifications to the standard protocol.

3. 3. MATERIAL AND METHODS.

3. 3. 1. Fixation and storage.

Acropora embryos and larvae were fixed for 15-60 min in 3.7% formaldehyde in Millipore-filtered seawater (MPFSW) buffered to pH8.0 with Hepes buffer. Fixed material was washed repeatedly in MPFSW, dehydrated through a graded methanol series and stored in absolute methanol at –20^{o}C until needed.

3. 3. 2. *In situ* hybridization and image capture.

Riboprobe synthesis and in situ hybridisation were performed as reported by (Hayward, et al. 2001). Following development embryos were washed in PBT and gradually dehydrated to 70% glycerol in which they were stored. They were mounted on microscope slides in 90% glycerol for photography. Digital images were captured with a Spot Camera mounted on a Wild Photomakroskop. Micrographs of embryos of varying stages were moved to a common white background and color and contrast adjusted for better visualization of staining patterns.

3. 3. 3. Sequencing.

DNA sequencing was performed using Big Dye Terminator v. 3.1 (Applied BioSystems) with vector and internal primers. Reactions were run on an ABI 3730 sequencer at the Biomolecular Resource Facility (JCSMR, ANU). Sequence anaysis was carried out using MacVector 9.5.2 (Accelrys) and Lasergene (DNASTAR).

3. 3. 4. RACE.

A 520 bp sequence with similarity to galaxin was identified in a screen for genes which are differentially expressed during metamorphosis (unpublished). 5’ and 3’ RACE was carried out with primary polyp RNA following the Clontech SMART RACE cDNA Amplification kit procedure. The primers used were: 5’RACE: 5’GATGGTGCTGTTTTTGGTGAGGAC3’; 3’RACE: 5’CTGCTGTAGTGGTCGTGTCCTCAC3’. The PCR products were ligated into pGEM-T Easy (Promega). The predicted cDNA sequence was assembled from the

overlapping 5' and 3' sequences using SeqMan (DNASTAR). The existence of the predicted transcript was confirmed by using the following 5' UTR and 3' UTR primers to generate a product of the predicted size and sequence: 5'UTRprimer: 5'AACAGAGGAGATAGCTAGTGT3'; 3'UTRprimer: 5'GTCCTCCTCGAGGTTACATCAC3'.

3. 3. 5. Virtual northern blots.

"Virtual northern blots" were made using the Clontech SMART cDNA Synthesis Kit, according to the manufacturer's instructions using RNA from the stages indicated.

3. 4. RESULTS.

3. 4. 1. Identification of three *galaxin*-related genes in *Acropora*

Two unigenes identified during EST analyses of *Acropora millepora* gave strong matches with *Gfgalaxin*, the gene coding for a soluble protein identified as a major component of the exoskeleton of the coral *Galaxea fascicularis* (Fukuda et al., 2003, Watanabe et al., 2003). One of these *(Amgalaxin*) is assumed to be the *Acropora* ortholog of Gfgalaxin, as the predicted proteins had 58% identity and 73% similarity overall (BlastP significance = $1e^{-105}$). The second unigene, *Amgalaxin-like 1*, encodes a larger protein with much lower overall identity to Gfgalaxin (BlastP significance = $7e^{-19}$). An additional related unigene, *Amgalaxin-like 2*, was identified in a screen for genes differentially regulated at the time of settlement, and has a BlastP significance against Gfgalaxin of $2e^{-33}$.

3. 4. 2. Structure of the predicted galaxin-related proteins from *Acropora*

The *Acropora* and *Galaxea* galaxins are very similar in most structural characteristics. Like its *Galaxea* counterpart, Amgalaxin is cysteine-rich (12.4% overall, compared to 13.4% in Gfgalaxin). The 338 AA *Acropora* predicted protein (Fig. 1A) consists of an N-terminal signal peptide of 23 AA, the bulk of the rest of the protein consisting of tandem repeats, each of around 30 AA and containing two di-Cysteine motifs (Fig 2A). Both proteins have a dibasic recognition site for processing endoproteases giving

predicted mature proteins of 298 amino acids (Gfgalaxin) and 292 amino acids (Amgalaxin).

The proteins are also similar in terms of predicted isoelectric points; for the mature proteins, pI values of 4.52 (Amgalaxin) and 4.53 (Gfgalaxin) were calculated. Gfgalaxin is a glycoprotein (Fukuda et al., 2003); however, only one of the two potential N-glycosylation (i.e. N-X-S/T motif) sites identified in Gfgalaxin is conserved in the *Acropora* protein (Asn178). The high content of threonine and serine residues (14% and 8.9 % in Gfgalaxin and Amgalaxin, respectively) suggests that O-glycosylation may be more significant. However, NetOGly (http://www.cbs.dtu.dk/services/NetOGlyc/) predicts only two positions with significant likelihood of O-glycosylation in Amgalaxin and a single potential site in Gfgalaxin.

3. 4. 3. The Amgalaxin-like proteins are related but divergent

The cDNAs encoding both of the Amgalaxin-like proteins are larger than that encoding Amgalaxin, as are the predicted proteins themselves; Amgalaxin-like 1 is predicted to be 660 AAs and Amgalaxin-like 2 is 582 AAs. The Amgalaxin-like precursor proteins have a common domain structure that differs from the galaxins by the presence of a domain rich in acidic amino acid residues (Fig. 1B and C). As in the case of the galaxins, signal peptides (24 and 16 AA in Amgalaxin-like 1 and 2 respectively) and Cys-rich repeat regions are present. Although the repeat units in the Cys-rich domain of Amgalaxin-like 1 are approximately the same size (29 AAs) as those in the galaxins, one difference that is potentially significant with respect to protein folding and higher order structure is the spacing of the cysteine residues.

Whereas the galaxin proteins have two di-Cys motifs per repeat unit, Amgalaxin-like 1 has only one. The other two cysteine residues in each repeat are separated by three amino acids. The basic repeat unit in Amgalaxin-like 2 is 41 amino acids long and contains three di-Cys motifs. As in the case of Amgalaxin-like 1, the repeats in Amgalxin-like 2 are more similar to each other than are the Amgalaxin repeats. When the consensus sequences of the Cys-rich repeats of the three proteins are compared it is apparent that Amgalaxin-like 2 and Amgalaxin share the greatest

similarity (Fig. 2). Although consensus sequences can be constructed for the galaxin and Amgalaxin-like repeats there is little similarity between the proteins apart from the arrangement of the dicysteine residues.

Amgalaxin and Amgalaxin-like 2 are both predicted to be moderately acidic proteins (pIs of 4.52 and 4.6 respectively for the mature proteins), whereas the mature Amgalaxin-like 1 protein is more basic (predicted pI of 9.09). The mature Amgalaxin-like proteins contain 13% and 16% Aspartic (Asp) and Glutamic (Glu) acid residues, predominantly located in one part of the protein, the acidic domain (Fig. 1B).

NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) predicts N-glycosylation at Asn48 in Amgalaxin-like 1 with high probability, but does not predict glycosylation at the single N-X-S/T site in Amgalaxin nor at either of the potential sites in the *Galaxea* protein. NetOGlyc (http://www.cbs.dtu.dk/serv.ices/NetOGlyc/) does not predict any likely O-glycosylation sites in Amgalaxin-like 1. The mature Amgalaxin-like 2 protein contains three potential N-glycosylation sites and several potential sites for O-linked glycosylation.

3. 4. 4. Expression analysis of *A. millepora galaxin*-related genes.

Virtual northern analysis shows that the *Amgalaxin* and *Amgalaxin-like* genes each have distinct temporal expression patterns (Fig. 3). We will therefore consider these genes in the order in which they are expressed. *Amgalaxin-like 1* is expressed strongly in pre-settlement planulae and primary polyps, but the mRNA was not detected in adult colonies (Fig. 3A). *Amgalaxin-like 2* expression is restricted to post-settlement polyps (Fig. 3B). By contrast, as in the case of its *Galaxea* counterpart (Fukuda *et al.,* 2003), *Amgalaxin* is expressed in both of the post-metamorphosis stages examined: weakly in primary polyps, but strongly in adult colonies (Fig 3C). In the case of *Amgalaxin*, two transcripts were detected on virtual northern blots (Fig 3C). The strong upper band, with an apparent size of approximately 2Kb, corresponds to the sequence shown; the lower (~1.7Kb) band is assumed to correspond to an alternatively spliced product.

Amgalaxin-like 1 spatial expression is first detected in a subset of aboral ectodermal

cells of the mid-late planula (Fig 4A-C, arrows), in a region previously described for a variety of Anthozoarians known as the "apical organ" which is believed to be involved in substrate recognition (Vandermeulen, 1974; Chia and Koss, 1979). During settlement and metamorphosis, *Amgalaxin-like 1* expression remains restricted to this ectodermal cell population (Fig 4D-I). At this time, changes in the overall morphology of the larva can be observed as it flattens down onto the substratum and mesenteries are formed. After the larva has flattened (Fig 4D), the *Amgalaxin-like 1* expression domain expands radially from the former aboral end of the planula to near the edge of the now flat primary polyp (Fig 4H-I). This outer, sub-marginal ring of expression on the aboral side persists until about the 12-septum stage before gradually dissipating. Throughout this time the expressing cells are distinct from one another and maintain a columnar morphology (Fig 4 A-H).

Consistent with the developmental virtual northern blot (Fig. 3B), *Amgalaxin-like 2* expression is not present in the pre-settlement planula larva (Fig. 5A-B), first appearing as the spindle-shaped planula shortens into a sphere (Fig. 5C-D) before flattening against the substratum (Fig. 5E-J;). Thereafter, expression is localized to an expanding ring in the aboral ectoderm (Fig 5C-J), in its final stages (Fig 6H-J) resembling the expression of *Amgalaxin-like 1* (Fig 5I).

Although virtual northern blot analysis showed that *Amgalaxin* expression was much stronger in the adult, expression was also present in immediate post-settlement stages. This finding is consistent with in situ hybridization results, which show expression beginning in an aboral ring resembling those seen for *Amgalaxin-like 1* and *2* (Fig 6A). However, there is one marked difference between *Amgalaxin* and the *galaxin-like* transcripts in that *Amgalaxin* is expressed along the calcifying septa from shortly after their initiation (Fig 6B-F) and was still apparent there at the twelve septum stage in the oldest polyp which we attempted to stain (Fig 6F).

3. 5. DISCUSSION.

All three of the *Acropora* galaxin-related proteins contain cysteine-rich domains including tandemly repeating di-Cys motifs (Fig. 1-2). The presence of these domains is consistent with these proteins having a structural role in the organic matrix, as

framework proteins are frequently Cys-rich (Shen *et al.,* 1997; Gotliv *et al.,* 2005) and tandem repeats with conserved Cys residues are commonly found in many types of extracellular matrix and cell surface receptor proteins (Shen *et al.,* 1997; Bhattacharya *et al.,* 2002). Double Cys motifs may form intramolecular cross-links via disulfide bonds (Shen *et al.,* 1997). However, in vitro studies with synthetic peptides indicate that dicysteine motifs may efficiently form cyclo-cystine loops (Liff and Zimmerman 1998), limiting their ability to form intra- (or inter-) molecular cross links (Watanabe *et al.,* 2003). Fukuda et al. (2003) suggest that therefore only the two terminal Cys residues may be available for intermolecular disulfide bonds.

Although only distantly related to the scleractinian galaxins, proteins with similar properties have been identified as components of the organic matrix of molluscs. For example, di-Cys motifs are found in lustrin A from *Haliotis rufescens* (Shen *et al.,* 1997), the N14 protein from *Pinctada maxima* (Kono et al, 2000) and in pearlin from *Pinctada fucata* (Miyashita et al., 2000). These similarities raise the possibility of common functions in biomineralization (Fukuda *et al.,* 2003; Watanabe *et al.,* 2003), either in establishing an overall structural framework onto which other matrix components may be deposited or in actively controlling crystal nucleation and development.

3. 5. 1. The Amgalaxin-like proteins combine characteristics of two distinct protein families involved in mollusc calcification.

Although Amgalaxin-like 1, Amgalaxin-like 2 and Amgalaxin are clearly related, one major difference is the presence of an acidic domain in the galaxin-like molecules that is absent from both the *Acropora* and *Galaxea* galaxin proteins. Proteins rich in acidic amino acid residues are important components of the organic matrices of a number of taxonomically diverse calcifying invertebrates. For example, Gotliv *et al.,* (2005) identified a family of Asp-rich proteins associated with the mineral component of the bivalve *Atrina rigida* and suggested roles in controlling mineralization.

Ameye *et al.,* (1999) identified an acidic N-glycoprotein in the organic matrix of spicules from the sea urchin *Paracentrotus lividus* and Rahman *et al.* (2005; 2006a,b) found acidic proteins that bind calcium in the organic matrix of spicules of the

alcyonarians *Sinularia polydactyla* and *Lobophytum crassum* and reported that both species possess aspartic acid rich matrices. Although there are no direct precedents from scleractinians, Puverel *et al.* (2005) found that matrices of both *Stylophora pistillata* and *Pavona cactus* were high in acidic amino acids (45.9 and 65.5% acidic amino acids, respectively) and obtained an internal sequence from *S. pistillata* that contained a long (36 residue) poly-Asp domain.

While the overall pI value for Amgalaxin-like 1 mature protein is 9.09, charged amino acid residues are distributed in a non-uniform way: the values for the acidic and Cys-rich regions are 3.21 and 12.39, respectively, and the Amgalaxin-like 2 protein has a similar bipartite structure. The pIs of the acidic domains are in the same range as those reported for some acidic protein components of molluscan matrices (Gotliv *et al.,* 2005), while the pIs of the Cys-rich regions resemble those of a family of matrix proteins from the pearl oyster *Pinctada fucada* (pI values of 9.5 and 9.8; Zhang *et al.,* 2006a).

The Amgalaxin-like proteins therefore combine characteristics that in molluscs reside in two distinct families of matrix proteins; an Asp-rich family of proteins (Gotliv *et al.,* 2005) which might actively control mineralization (Fukuda et al., 2003), and a family of basic proteins that could be involved in linking hydrophilic molecules to the framework of the organic matrix, or binding carbonate (Zhang *et al.,* 2006).

The acidic domains found in the AmGalaxin-like proteins are likely to control basal plate deposition by actively binding calcium. Gotliv *et al.,* (2005) reported a calcium-binding domain similar to that from Calsequestrin in the Asp-rich proteins and mentioned that this type of calcium-binding activity is related to the negatively charge carboxyl groups from Asp or Glu residues. Furthermore, Addadi *et al.,* (2006) reported that acidic domains were occluded into the crystal phases of mollusc shells and Nudelman *et al.,* (2006) reported areas rich in carboxyl groups from Asp and Glu side chains underlying newly form $CaCO_3$ crystals while crystal growth areas contained few acidic functional groups. Although Gotliv *et al.,* (2005) mentioned that to date only acidic matrix proteins are associated with calcite deposition, Nudelman *et al.,* (2006) reported and assemblage of acidic proteins in the OM from *Nautilus pompilius* that are able to nucleate aragonite.

3. 5. 2. What is the function of the Amgalaxin-like proteins?

Although the galaxin-like proteins have an overall structural similarity, they are otherwise dissimilar at the primary amino sequence level, and may be expected to fulfill somewhat different functions during calcification. Their sequential expression during development (Fig 3) is consistent with this suggestion.

Deposition of the coral skeleton is thought to occur as a two-step process; the first step is the formation of early mineralization zones characterized by the presence of randomly oriented grains of $CaCO_3$, followed by a second step characterized by crystal-like fibers of aragonite. This pattern of mineralization has been described in newly settled *Pocillopora damicornis* (Vandermuelen and Watabe, 1973) and *Porites porites* (Goreau and Hayes, 1977) and in axial polyps of *Acropora cervicornis* (Gladfelter, 1982). Although these two kinds of calcification probably both occur throughout the coral life cycle (Cuif and Dauphin, 2005), several lines of evidence suggest that granular calcification predominates during early coral development, whereas calcification may be largely of the fibrous type in adult colonies. However, it is only in newly settled polyps that the two processes can be studied in isolation.

Goreau and Hayes (1977) describe the beginnings of skeleton formation as the secretion of "mucoid substances" which serve to cement the settling planula to the substratum and it is possible that this could be the function of the Amgalaxin-like 1 protein. In contrast, in their descriptions of the early post-settlement stages Vandermulen and Watabe (1973) and Le Tissier (1988) concentrated on the calcified elements of the early skeleton, perhaps because of the preparation techniques that they used. The earliest post settlement stage of *Pocillopora* shown by Le Tissier (1988, Fig. 2a) has a complete circular rim of calcified material on the basal plate with scattered areas of calcification within this circle, while in *Porites* Goreau and Hayes (1977, Fig. 4-2) show a portion of the rim of the plate as calcified with scattered areas of calcification on the plate.

Clode and Marshall (2004) measured intracellular calcium in pre- and post-settlement larvae of *Pocillopora damicornis* using Calcium Orange fluorescence and found that

levels of intracellular calcium were lowest in a small area at the aboral end of pre-settlement and immediate post-settlement planulae. This area corresponds extremely well to areas of *Amgalaxin-like 1* expression shown in Fig. 4A-E. Whether there is a functional relationship between the two patterns remains to be investigated.

The expression of *Amgalaxin-like 2*, in contrast, essentially demarcates the outer limits of the low Ca^{2+} area seen in *Pocillopora*. So, based on their respective distributions we suggest that the two Amgalaxin-like proteins are engaged respectively in laying down the organic matrix in advance of calcification and in the process of calcification itself in the case of Amgalaxin-like 2. Another possibility is that transiently expressed proteins such as the Amgalaxin-like molecules may actually be inhibiting the formation of aragonite fibers in the basal plate while inducing calcite deposition. Indeed, there is an invertebrate precedent for such an action of acidic proteins since Addadi and Weiner (1985) established that they could alter the morphology of calcite crystals by adding an acidic matrix protein from the bivalve mollusc *Mytilus*.

3. 5. 3. Amgalaxin and the mesenteries: fiber-like calcification.

Following its initial expression in an aboral ring resembling those seen for the *Amgalaxin-like* transcripts (Fig 6A-B), *Amgalaxin* is expressed along the calcifying septa in a pattern distinct from the *Amgalaxin-like* transcripts. The close correspondence between the appearance of mesenteries (and the implied initiation of septal mineralization) and the initiation of *Amgalaxin* expression is consistent with involvement of Amgalaxin-like in deposition of the basal plate and protosepta, Amgalaxin could thus be involved in the fiber-like calcification characteristic of mesenteries.

The strong expression of *Amgalaxin* in adult *Acropora* and the abundance of the corresponding protein in mature *Galaxea* colonies (Fukuda *et al.,* 2003) imply that galaxins may be involved in controlling the fiber-like aragonite deposition characteristic of adult skeletons. According to Vandermeulen and Watabe (1973) the basal plate of *Pocillopora* contains a mixture of calcite and aragonite, so neither the circular expression pattern nor septal expression is inconsistent with the idea that the

Amgalaxin-like molecules are associated with granular calcification and Amgalaxin with aragonite. Aragonite deposition greatly predominates in the adult coral and only Amgalaxin expression persists into that stage.

As in the case of mucoperlin, a component of the mollusc organic matrix (Marin *et al.,* 2000), galaxins resemble mucins in that they have a high serine content with consequent glycosylation potential and have a tandem repeat structure. This structure is consistent with the idea that these and other ECM genes may have evolved from a mucin-type ancestor (Marin *et al.,* 1996). However, the experiments of Fukuda et al. (2003) indicate that Gfgalaxin does not bind Ca^{2+}, so the exact role of all of the galaxin-related proteins remains to be established.

3. 5. 4. Galaxins as examples of functional convergence after secondary recruitment?

Massive calcification is a widespread trait across the animal kingdom; obvious examples are found among echinoderms, molluscs, vertebrates and corals. Many lines of evidence indicate that the trait has evolved independently in these lineages despite the involvement of some common classes of molecules (e.g. carbonic anhydrases). Although the coral galaxins and the mollusc lustrin A/N14/pearlin proteins may have separate evolutionary origins, their structural similarity suggests common function. Likewise the galaxin-like proteins may combine the functions of two distinct classes of mollusc proteins – the acidic proteins that are thought to actively control mineralisation, and basic proteins that either bind bicarbonate or form links with the organic matrix.

Although proteins containing multiple di-Cys motifs are widely distributed, clear orthologs of galaxin are so far known only from scleractinians. However, there are predicted proteins in the non-calcifying cnidarians *Nematostella* (e.g. EDO38853.1) and *Hydra* (XP_002169304; "usherin-like") having moderate degrees of similarity to the scleractinian galaxins, and a "galaxin" has been reported in the vestimentiferan tubeworm *Riftia pachyptila* (Sanchez et al., 2007). There are also database entries annotated as either "similar to galaxin" or "galaxin-related" from *Ciona* and *Oikopleura* (AAS21342), respectively.

The product of the *Ciona* ("similar to galaxin") gene ci0100148033 gives a BlastP score of $2e^{-31}$ against Amgalaxin (for comparison, BlastP similarity between Amgalaxin and Amgalaxin-like 1 is $4e^{-29}$) and the gene is expressed throughout the epidermis at the late tailbud stage (Aniseed database: http://aniseed-ibdm.univ-mrs.fr/insitu.php?id=2732806), while the *Riftia* "galaxin" was identified as body-wall specific in differential display experiments (Sanchez *et al.*, 2007). Together with the *Acropora* data, these various lines of evidence suggest that the ancestral galaxins may have been structural ECM proteins that were secondarily recruited to roles in skeletogenesis during the Triassic, when scleractinians first appear in the fossil record. Comparison of sequences and expression data across a broad range of cnidarians and other animals will be required to clarify the evolution of this protein family.

3. 6. CHAPTER FIGURES.

Figure 3. 1. Inferred protein sequences of Amgalaxin and the Amgalaxin-like molecules.
Amgalaxin differs from the galaxin-like molecules in that it lacks an acidic domain. The signal peptide is marked in green, the acidic domain in yellow, and the di-Cys repeats in red. Potential N-linked glycosylation sites in Amgalaxin and Amgalaxin-like-2 are shown in blue.

(A) Amgalaxin

```
54:   SCGGVLYNPAAEMCCHGNVEPRVGASPM
82:   CCESSSYDPSTQMCCEGTVSNKPPGIAM
110:  CCGSEAYDANSQICCNGNINTKATGPTAQPG
141:  CCGEFSYDAASQLCCDSHPVLMVGSLPS
169:  CCGRNGYDANTSLCCGDNNVAFVSGPQAA
198:  CCGDMGYNRNTHLCCDSNVLPMPAMGA
225:  CCGSWTYSQQTHLCCEGVQLYKGMNTG
252:  CCGAVGYNQVNSLCCEGTVVPKSPSKPV
280:  CCGTTSYNPLTELCCDGIAFFKTGFIRPT
309:  CCGGAIYDATVARCCDGVPTYNVASCAGLA

Consensus
      CCG...YD..T.LCC.G....K.....
```

(B) Amgalaxin-like 1

```
223: CGRIRYIPSKQRCCN-RRVIPRHLPCKPK
251: CRTKYYNPYSHKCCFGRIVTPKPRPCLLG
280: CGRRYYNPLTHKCCFGRVVTPRSRPCRSV
309: CGSKSYNPLTHRCCFGRVVTSKLRHCPLR
338: CGVRYYNPLTHKCCFGRVVTPRSRPCSRL
367: CGSKYYNPLTHKCCFGRVVTPKLRPCLLR
396: CGVRYYNPLTHKCCSGRVVTPRSSPCRTV
425: CGLKYYNPLTHRCCFGRVVTPKSRPCPLR
454: CGVRYYNPLTHKCCFGRVVTPRLRPCLLR
483: CGVRYYNPLTHKCCFGRVVTPRLRPCLLR
512: CGVRYYNPLTHKCCLGRVVTPKLRPCLLR
541: CGVTSYNPITQKCCSRRLVISKLRPCPLK
570: CGSRYYYPTTQKCCFGHVVKPKTSPCLLR
599: CGLKFYNPLTFKCCVGNIVTPKPFSCPLP
628: CGATYYKPVSHRCCYGTVILKSFK-CVIP

Consensus
     CG..YYNPLTHKCCFGRVVTPK.RPCL
```

(C) Amgalaxin-like 2

```
309: CCGSSVYTIGTQLCCSGRVLTKNSTINA--CCGTQGYNIRTHR
350: CCGRTLYNRNTQLCCQGRILPKRSTINA--CCGTQGYNIRTHR
391: CCRRTLYNRNTQLCCQGRIISKNSTINA--CCGTQGYNIRTHR
432: CCGRTLYNRNTQLCCQGRIISKSSTINA--CCGTQGYNISTHS
473: CCRRTLYNRNTQLCCYGRIHAKTLSRKTRLCCGSSSYTTTTHL

Consensus
     CCGRTLYNRNTQLCCQGRI..K.STINA  CCGTQGYNIRTHR
```

Figure 3. 2. Alignments of the Cys rich repeats of the three proteins.
Numbers on the left indicate the position in the predicted protein. A consensus sequence for the repeats from each protein is shown beneath the alignments. Residues are included in the consensus if they are represented in at least 50% of the repeats.

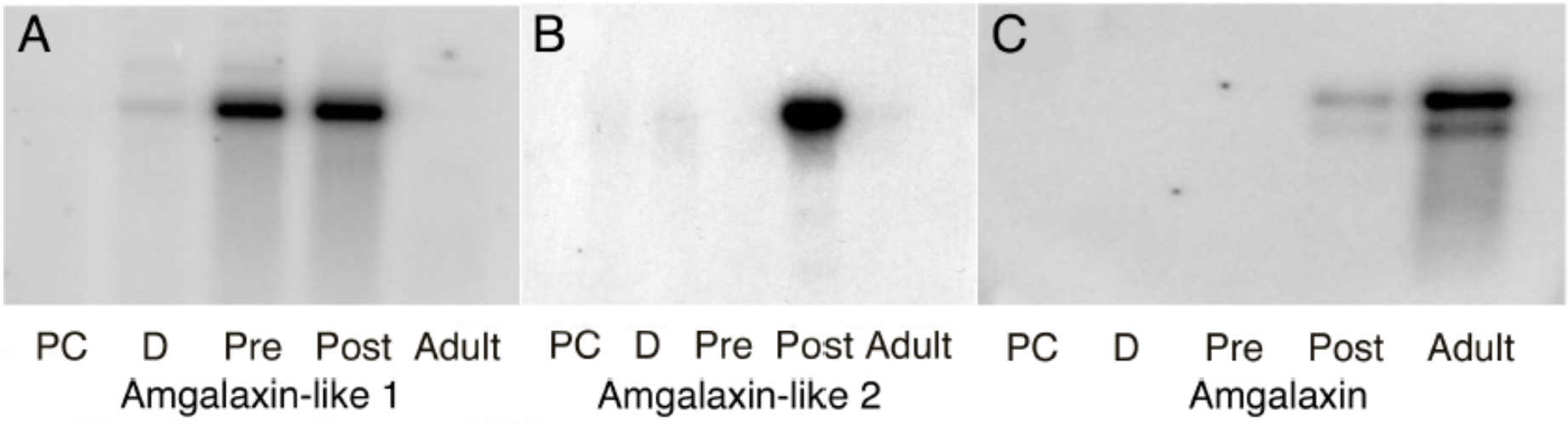

Figure 3. 3. Virtual northern blots of the three genes arranged in the order in which they are expressed.
The various stages represented on each blot are labelled across the bottom of the blot. *Amgalaxin-like 1* expression first appears weakly even before gastrulation and is strong both before and after settlement, but is not apparent in the adult. *Amgalaxin-like 2* is expressed exclusively immediately after settlement. *Amgalaxin* apparently has two isoforms both of which are expressed weakly immediately after settlement and more strongly in the adult. Abbreviations : PC=prawn chip, D=donut, Pre=presettlement planula larva, Post=polyp immediately after settlement.

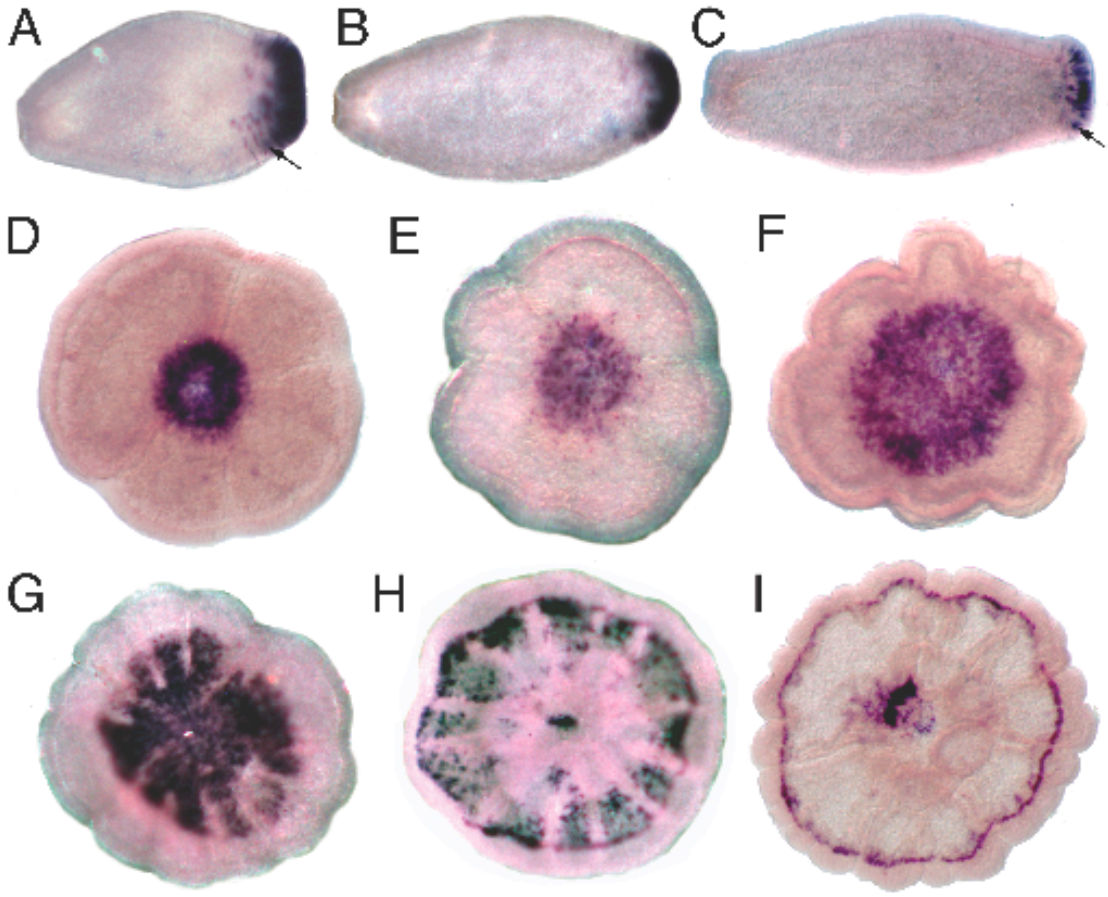

Figure 3. 4. Developmental expression of *Amgalaxin-like 1.*
(A-C) Expression begins in a zone of strong expression at the aboral end of the planula. At the margins of this zone the expressing cells are no longer contiguous (arrows). (D-F) After settlement the zone of expression on the aboral side of the polyp expands as the polyp ages. (G-H) The zone of expression then begins to fragment segmentally, eventually leaving a few traces of expression centrally as well as a submarginal ring of expression (I).

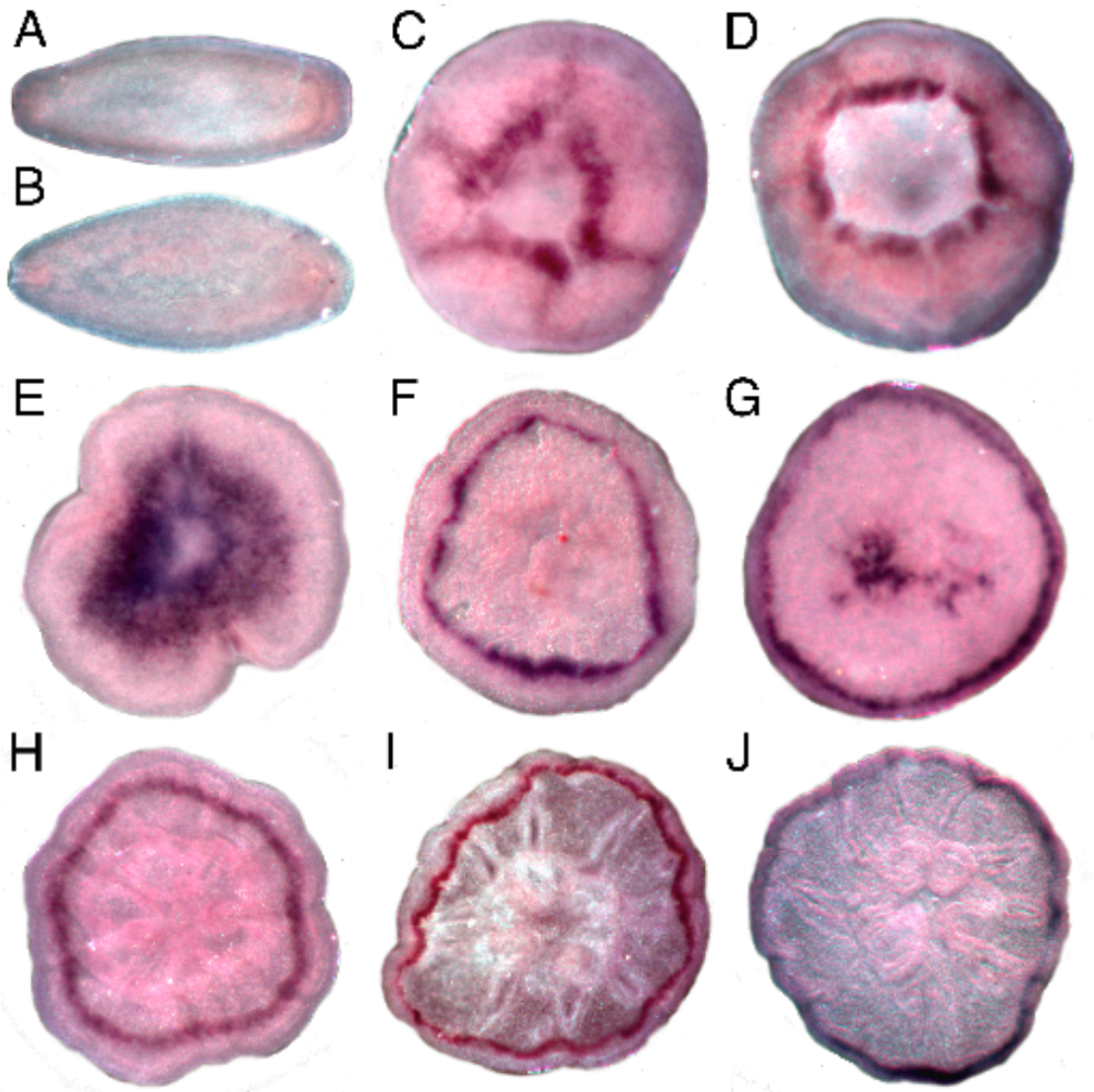

Figure 3. 5. Developmental expression of *Amgalaxin-like 2.*
There is no expression in the planula larva (A-B). Expression first appears as a ring on the aboral side of the planula as it shortens to form a sphere (C-D). Expression continues aborally, sometimes in a zone (E) and sometimes in a submarginal ring (F-J) as the polyp ages. In F,H and J the polyp is viewed orally and the aboral expression is seen through the cleared tissue.

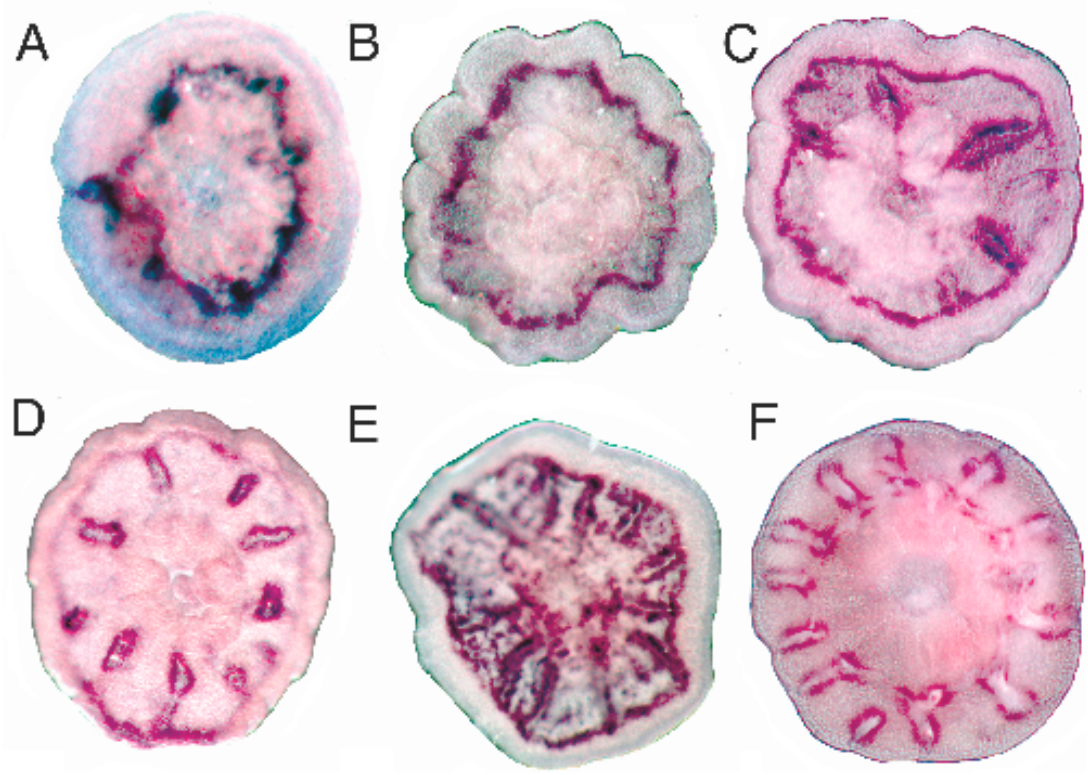

Figure 3. 6. Developmental expression of *Amgalaxin*.
Expression begins as an aboral submarginal ring (A-B) Septal expression is then added (arrows) as the polyp grows older (C-F). There is also sometimes aboral granular expression between the septa (E). Septal expression continues in the oldest polyp studied (F).

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CHAPTER 4. An atypical calmodulin and a neuronal calcium sensor protein as potential targets of CaM regulation during development of the coral *Acropora millepora*.

4. 1. ABSTRACT.

Changes in cytosolic calcium concentration regulate a wide variety of processes in eukaryotes including apoptosis, cell differentiation and the secretory pathway. Many of the mechanisms of calcium regulation are conserved across distant phylogenetic groups, as are a number of the proteins involved in calcium homeostasis. Calmodulin (AmCaM) and neurocalcin (AmNC) orthologs were identified in *Acropora millepora*; these, and a coral specific calcium sensor protein (AmCaM-like) were expressed through early development, indicating potential roles in regulating coral metamorphosis and early stages of calcification. The AmNC and AmCaM-like proteins contain internal CaM-binding sites and were shown to interact *in vitro* with AmCaM. These results suggest that both conventional calcium dependent signalling pathways and coral specific modifications of a common scheme might regulate coral specific processes such as metamorphosis and skeleton deposition.

4. 2. INTRODUCTION.

Eukaryotic organisms use changes in intracellular calcium concentration as a secondary messenger to regulate a variety of cellular signalling pathways such as fertilization, cell division, metamorphosis, and apoptosis (Haeseleer *et al.,* 2002; Carafoli, 2004). This is due to both the calcium ion's capacity to coordinate multiple molecules and the fact that its concentration gradient is 10,000 fold larger in the extracellular space than inside eukaryote cells (Carafoli, 2005; Parekh, 2006). Extracellular calcium enters the cell via voltage-gated, ligand–operated, and capacitative calcium channels. These intracellular calcium influxes are used to trigger different signalling pathways or to replenish depleted intracellular stores (Carafoli, 2004).

To avoid toxicity cytosolic free calcium concentration needs to be carefully regulated i.e. ~100-200nM (Carafoli, 2003). This is achieved by secreting excess calcium back

to the extracellular space, or stored it within membranes via calcium ATPases and sodium/calcium and hydrogen/calcium exchangers (Carafoli, 2004). Most of the calcium used to regulate cellular processes by eukaryote cells is released from cellular stores (Carafoli, 2004); this control mechanism allows the increase of calcium concentration in localized cellular regions for a limited period of time, which is a critical factor determining the initiation of specific intracellular signalling pathways (Carafoli, 2003).

Calcium homeostasis and signalling of precise cellular processes requires a number of intracellular control mechanisms able to regulate and coordinate cellular activity. This is achieved via calcium itself, which controls and auto-regulates the transcription and activity of its own transporters and channels via calcium binding proteins (Carafoli *et al.,* 1999; Carafoli, 2004). There are two types of calcium binding proteins: those that transport or buffer calcium, and those that are able to translate its signal. Most buffering proteins are acidic and have low calcium affinity, whereas those that translate calcium signals known as "calcium sensors" have high calcium affinity and experience topological changes that allow them to interact with target proteins (Carafoli, 2003).

According to Gerhart (1999), signalling pathways have been conserved during evolution, an idea supported by the presence of orthologs for most key vertebrate calcium signalling and homeostasis molecules in the *Acropora millepora* EST database. Furthermore, it is believed that modern CaM molecules evolved in the stem of eukaryotes from an ancestral CaM gene from which subsequent duplications allowed the diversification of calcium binding proteins and calcium dependent cellular pathways among eukaryotes (Baba *et al.,* 1984; Haeseleer *et al.,* 2002). CaM coding genes have been previously characterized in other Cnidarians (Jamieson et al., 1980; Yuasa et al., 2001) and are found in *Hydra capillata* and *Nematostella vectensis* EST databases.

Thus, is reasonable to think that coral CaM as well as specific calcium sensors are to some extent responsible for coral-specific process such as exocytosis of organic matrix precursors and the calcium transport/storage mechanisms necessary for skeleton deposition (Clode and Marshall, 2002; Puverel *et al.,* 2005) as well as tissue

rearrangement and calicoblast differentiation during metamorphosis (Vandermeulen, 1974; Clode and Marshall, 2004).

Hermatypic corals are responsible for much of the calcium carbonate deposition in coral reefs (Chave *et al.* 1975), one of the most productive ecosystems on earth (Gunderson, 2007); they belong to the class Anthozoa (Hexacorallia: Scleractinia), the most basal lineage within the phylum cnidaria, which is considered the sister group to Bilateria (Dunn *et al.,* 2008; Medina *et al.,* 2001). According to this, the study of calcium dependent cellular pathways in the coral *A. milllepora* not only will provide valuable information for the management and conservation of coral reefs in response to climate change and rising levels of CO_2, it also represents an ideal model to study the evolution and diversification of calcium dependent cellular pathways in the animal kingdom.

This study focuses on the characterization of EF-hand calcium sensors in the reef building coral *A. millepora* with emphasis on putative AmCaM interactions likely to coordinating vesicle transport and metamorphosis during settlement and early stages of coral calcification.

4. 3. MATERIALS AND METHODS.

4. 3. 1. Collection of larvae.

Acropora millepora colonies were collected from Magnetic Island, Queensland, Australia. Mature colonies were placed in large containers of seawater on the shore before dusk prior to spawning. After spawning, egg and sperm bundles were removed from the surface and mixed in containers containing fresh seawater. Fertilization lasted 2h. Developing embryos were maintained until reaching the desired developmental stage, at which time embryos were removed and frozen in liquid nitrogen for RNA extraction and cDNA synthesis. Every 12h, dead embryos and resulting surface lipids were removed and the water exchanged to allow for continued development.

4. 3. 2. RNA extraction.

Total RNA was extracted from *Acropora millepora* embryos collected at various stages of development. Extraction of RNA was achieved using Ambion RNAwiz™ RNA isolation reagent and associated protocols.

4. 3. 3. RNA quantification.

RNA quantification was determined spectrophotometrically using the BIORAD Smart Spec™ spectrophotometer at a wavelength of 260nm where an absorbance of 1.0 = 40μg/mL RNA.

4. 3. 4. Preparation of cDNA for Virtual Northern Blotting.

'Virtual' northern blots were used to assess the developmental expression pattern of the genes of interest due to the scarcity of material available to make repeated batches of RNA. First strand cDNA was synthesized using the BD Biosciences SMART PCR cDNA synthesis kit according to the manufacturers specifications.

4. 3. 5. Southern blotting and hybridisation.

cDNA was transferred from agarose gels to nylon membranes as described in Sambrook *et al.* (1989) and Amersham membrane protocols. The DNA of interest was electrophoresed on an agarose gel and transferred to Nytran-N (Schleicher & Scheull) or Hybond-N, -N+ or -NX (Amersham) using the capillary blot transfer method overnight in 20xSSC or 0.4M sodium hydroxide (Hybond-N+). DNA was covalently linked to membranes by baking at 80°C for 2h.

Following fixation of DNA, membranes were prepared for hybridisation by incubating in 100 ml of DNA hybridisation solution at 60°C for 2 - 3 h. The DNA was hybridized to the membrane at 60 - 65°C for at 12 - 16 h, after which it was washed (2 x 15 min) with 200 ml of low stringency wash solution (2xSSC; 0.1% SDS) at 60 - 65°C. Membranes were monitored with a Geiger counter and exposed to Phosphorimager screens for 3 - 5 h. Screens were scanned using a Phosphorimager (Molecular Dynamics) and images were processed using ImageQuant software (Molecular Dynamics).

4. 3. 6. Generation of radioactive probes.

Radioactive probes were prepared by random oligonucleotide-primed synthesis (oligolabelling) using α-^{32}P dATP (Geneworks; 10 mCi/ml, specific activity ~3000 Ci/mmol). Generally, 25 ng of linear DNA recovered from agarose gel fragments were radioactively labelled using the Megaprime (Amersham Biosciences) or Prime-a-Gene (Promega) oligolabelling kits as described in the accompanying protocols. After labelling, unincorporated α-^{32}P-dATP was separated from the labelled probe by spermine precipitation. Precipitation of the labelled probe was achieved by adding spermine to a final concentration of 7.5 μM and incubating on ice for 20 min before centrifugation at 15,000 g for 15 min. The supernatant containing unincorporated label was removed and the probe resuspended in 100 μl probe resuspension solution (10 mM EDTA; 0.5% SDS). Prior to use, the labelled probe was denatured at 100°C for 5 min and placed on ice to prevent re-annealing. Following the ice incubation, probes were immediately added to the hybridisation solution.

4. 3. 7. Fusion-protein expression.

BL21 *E. coli* competent cells were transformed with either pGex-DMZ+1.8 or pProEXb expression plasmids containing the selected coral cDNA. Once positive transformants where identified by PCR, restriction enzyme digestion and sequencing, the selected clones were used to initiate small-scale overnight bacterial cultures. The following morning, 2.5 ml of the overnight culture were used to inoculate 250 ml of fresh LB medium + Ampicillin (100μg/ml) and cultured until the optical density at 600nm reached 0.5-0.8. At this stage protein expression was induced by adding IPTG to a final concentration of 1mM and incubated for 3 hours. Cultures were then harvested by centrifugation at 4^0C at 4000 rpm/15min. Pellets were suspended in 10ml of ice cold PBS and lysed by sonication. Cell debris were pelleted at 10.000x/5min and supernatants subjected to protein purification.

4. 3. 8. Glutathione Sepharose affinity purification.

Equilibrated 50% Glutathione Sepharose 4B suspension beads (Pharmacia Biotech) were added (0.25ml) to recovered supernatants and purification carried out according to the manufacturer's manual. The mixture was incubated at room temperature for 30min with gentle rotation after which mixtures were loaded into purification columns and washed 3x with 5ml ice-cold PBS. Fusion-proteins were eluted by addition of 0.5ml of 10mM reduced glutathione in 50mM Tris-HCL buffer (pH 8.0). Eluted samples were subjected to standard protein electrophoresis.

4. 3. 9. Ni-NTA affinity purification.

250 μl of Ni-NTA resin were added to recovered supernatants and purification carried out according to the manufacture's manual (QIAGEN). Mixtures were incubated at 4^0C for 1 hour with gentle shaking and then loaded onto purification columns. Samples were washed 3x with wash buffer and eluted by adding 0.5 ml of elution buffer. Both wash and elution buffers were supplied by the manufacturer (QIAGEN). Eluted samples were subjected to standard protein electrophoresis.

4. 3. 10. Polyacrylamide gel electrophoresis.

After protein purification, 20ul of sample were mixed with 20ul of loading buffer (50mM Tris-HCL pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% Glycerol) and fractioned in a 10%- 12% acrylamide (acrylamide:N,N'-methylene-bis-acrylamide=29:1)/0.5x TBE (Tris-borate-EDTA) gel according to Sambrook *et al.,* (1989). Gels were stained with Coomassie brilliant blue and photographed. Fusion-protein size was estimated by using standard commercial protein markers.

4. 3. 11. Affinity chromatography.

GST-calmodulin fusion protein was expressed and purified in the presence of 1mM $CaCl_2$ and 5mM EGTA in both wash and elution buffers. Putative coral calmodulin-interacting His fusion-proteins were expressed and purified as descried previously. GST-AmCaM fusion protein-soluble sonicated samples were incubated with either

purified His-AmCaM-likeB or His-AmNC fusion proteins and Glutathione Sepharose matrix in the presence of 5mM EGTA and 1mM $CaCl_2$ for one hour at 4°C shaking.

After this time mixtures were loaded onto a protein purification column and the flow through collected. Once the Glutathione Sepharose matrix was washed 3x with PBS containing either 5mM EGTA or 1mM $CaCl_2$ the GST-AmCaM fusion protein was eluted with reduced glutathione buffer containing either 5mM EGTA or 1mM $CaCl_2$. Eluted and flow through samples were ran on a standard 10% SDS-PAGE to test for co-localization of AmCaM and its putative His-Tagged target fusion protein.

In a similar way, sonicated soluble samples containing His-tagged putative targets fusion-proteins were incubated with GST-AmCaM and Ni-NTA resin in the presence of 5mM EGTA and 1mM $CaCl_2$ for one hour at 4°C with shaking. After this time mixtures were loaded onto a purification column and the flow through collected. Ni-NTA resin matrix was washed 3x with PBS buffer containing either 5mM EGTA or 1mM $CaCl_2$, and His-tagged fusion proteins were eluted with buffer containing 250 mM Imidazole and either 5mM EGTA or 1mM $CaCl_2$. Once again eluted and flow through samples were subjected to standard protein electrophoresis in order to test for co-localization of calmodulin and its putative target proteins within the same fraction.

4. 3. 12. Western blotting.

Commercial rabbit polyclonal antibodies raised against amino acids 1-149 representing full-length human calmodulin I (CaMI) were purchased from Santa Cruz biotechnology and tested against coral calmodulin. Purified GST-calmodulin, GST-CaM-like fusion-proteins and coral CaM putative target proteins were run on two 10% protein gels with a similar loading pattern at 200-250 volts until the dye front reached the bottom of the gel. One of the gels was stained with Coomassie brilliant blue and photographed, while proteins resolved on the other were transferred from the polyacrylamide gel to a nylon membrane by electrophoretic transfer (Sambrook *et al.,* 1989). After transfer the polyacrylamide gel was stained with Coomassie brilliant blue in order to assess transfer efficiency.

Nylon membranes were washed 3X with tris-buffered saline (TBS) and sealed in a bag with 0.5% non-fat powder milk as blocking agent in 10ml of TBS for one hour. After that time 10µl of human CaMI antibody from a 200µg/ml stock solution were added to the sealed bag and incubated at room temperature over night with gentle shaking. The following morning membranes were washed 3x with TBS for 15min to remove unbound antibody.

Remaining primary antibody was blotted using rabbit-anti-mouse horseradish peroxidase (HRP) conjugated IgG. Unbound secondary antibody was removed by washing 3X for 30 minutes with TBS. Antibodies were detected using a chemiluminescent substrate that reacts with HRP to produce light in the visible spectrum after incubation for 5 minutes. The released light was detected by exposure of X-ray film, which was developed using traditional techniques.

4. 3. 13. Inmunoprecipitation.

Recovered supernatants from sonicated GST-AmCaM expression cultures were incubated with both purified putative His-AmCaM-like and His-AmNC fusion-proteins. Samples were incubated in the presence of 5mM EGTA and 1mM $CaCl_2$ at 4°C for one hour with gentle shaking. After this time 50µl of human CaMI agarose (25%) conjugated antibody from a 500µg/ml stock solution (Santa Cruz) were added to the protein mixtures and incubated for one hour at 4°C shaking. Samples were centrifuged and pellets washed 3x with 0.5ml of PBS containing either 1mM$CaCl_2$ or 5mM EGTA. Both pellets and subsequent washed samples were subjected to standard protein electrophoresis in order to identify co-precipitation of putative AmCaM target His-fusion proteins and GST-AmCaM in the same fraction.

4. 4. RESULTS.

4. 4. 1. Identification of three *Acropora millepora* EF-hand proteins.

A predicted protein set derived from early stage *A. millepora* cDNA libraries was scanned for the presence of EF hand domains (Pfam Id: PF00036), resulting in the identification of three cDNAs encoding putative calcium sensor proteins. Two of

these clearly correspond widely distributed proteins - a classic calmodulin protein, designated as AmCaM (Fig 1A) and a member of the neurocalcin (NC) family, known here as AmNC. The third calcium sensor protein is a divergent member of the calmodulin family, known here as AmCaM-like, and appears to be coral specific.

AmCaM, encodes an acidic protein of 149 amino acids with a predicted isoelectric point (pI) of 4.15 and a molecular weight (MW) ~17 kDa (Fig 1A). Similar to other CaM molecules the AmCaM protein contains four putative active EF-hand motifs (~13 amino acids long) evenly distributed along the full length of the molecule. Two EF-hand motifs are located in the N-terminal half of the protein and the remaining two are located in the C-terminal half. The protein is rich in acidic amino acids, i.e. Glutamate (14.8%) and Aspartate (10.7%)(Fig 1A).

In a similar way, the AmNC protein resembles other NC molecules (Fig 1C); it encodes a protein of 176 amino acids with a MW of ~20 kDa, has a predicted pI of 5.02 and is rich in acidic residues i.e. Glu (10.8%) and Asp (6.8%). In addition, similar to other NC orthologs AmNC possess only one N-terminal EF-hand and two C-terminal EF-hand motifs (Fig 1C). However, contrasting with its orthologs and based on conserved domain sequence similarity (Prosite) the last EF-hand in the AmNC protein is likely to be inactive. Despite this, AmNC showed high sequence identity with both vertebrate (76%) and invertebrate NC sequences, including that from the related anthozoarian *Nematostella vectensis* (82%). As a typical member of the Neuronal Calcium Sensor (NCS) family of proteins (Haeseleer *et al.,* 2002; Burgoyne, 2007), AmNC possesses a N-terminal myristoylation site likely to regulate the cellular localization of the protein in a calcium-dependent manner (Burgoyne and Weiss, 2001; Burgoyne, 2007).

Although AmCaM-like encodes an acidic protein of 150 amino acids with a predicted pI of 4.54 and a MW ~17 kDa that resembles AmCaM, conserved domain sequence similarity (Prosite) predicted that only the second EF-hand is likely to bind calcium. This prediction is based on substitutions in the EF-hand loop specially the 9^{th} residue. These changes can affect calcium-binding affinity up to 1000 fold (Burgoyne and Clague, 2003).

As indicated above, AmCaM-like has no clear orthologs in any organism, and at this stage is coral specific (1B).

4. 4. 2. Calcium signalling during coral development: conserved and specific calcium dependent pathways.

To investigate the temporal expression patterns of the *A. millepora* EF-hand proteins reported here, virtual northern analysis was carried out (Fig 2). Results indicated that the AmCaM and AmCaM-like transcripts were both ~ 1500bp (Fig 2B and 2C), whereas the AmNC transcript was larger (~2500 bp; Fig 2D). Each gene differed with respect to temporal expression pattern. AmCaM transcripts appeared to be present at a relatively uniform level across the range of developmental stages examined (Fig 2B); the relatively high levels present at the prawn chip stage imply a significant maternal contribution, as has been reported for various other animals (Floyd et al., 1986; Hanson-Painton et al., 1992; Knott et al., 2006).

The AmCaM-like transcript was first detected at the gastrulation stage, and its abundance appeared to increase throughout development, reaching its peak after settlement (Fig 2C). AmNC expression was first detected at late gastrulation, but was relatively constant from the pear stage through to post settlement (Fig 2D). These results indicate stage specific calcium dependent signalling pathways during coral development which function could vary from the canonical calcium homeostasis and house keeping roles and extend to stage specific processes such as metamorphosis and skeleton deposition.

4. 4. 3. Both AmCaM-like and AmNC interact with AmCaM *in vitro*.

Use of the Calmodulin Target Database (www.calcium.uhnres.utoronto.ca) allowed the identification of potential calmodulin-binding sites in both the AmCaM-like and AmNC proteins. In both cases 12 AA binding sites were predicted between EF-hand motifs. While the predicted binding site in AmCaM-like is between EFIII and EFIV (Fig 1B), that in AmNC is predicted between EFI and EFII (Fig 1C).

The presence of predicted calmodulin-biding sites, and the fact that their expression temporally overlaps during the period of coral development spanning metamorphosis and the initiation of skeleton deposition, suggests AmCaM-like and AmNC might be subject to calmodulin-mediated regulation. To investigate their potential interactions *in vitro*, recombinant *Acropora* proteins carrying either GST or His-tags at their N-termini were produced by expression in *E. coli* BL21 cells and purified using the appropriate affinity ligand (glutathione agarose or Ni-agarose). In each case, the observed size of the fusion proteins on SDS-PAGE corresponded well with predicted values (Fig 3A).

Affinity chromatography and immunoprecipitation techniques were used to evaluate the ability of AmCaM to interact *in vitro* with AmCaM-like and AmNC in the presence of free calcium (1mM $CaCl_2$) or in its absence (5mM EGTA). Despite the series of washes (3X) performed to the loaded Ni-NTA resin/fusion-proteins complexes, when the immobilized His-tagged proteins were eluted in the presence of calcium from the Ni-NTA resin, GST-AmCaM was found in the eluted fractions with both His-tagged proteins (Figs 3B-3C). In the absence of calcium GST-AmCaM came out with the flow through and only His-AmCaM-like was observed in the eluted fraction (Fig 3B). On the other hand, in the absence of calcium His-AmNC failed to bind to the Ni-NTA resin matrix (Fig 3C).

In contrast with the affinity chromatography experiments using immobilized His-tagged proteins, when both His-AmCaM-like and His-AmNC fusion proteins were incubated (separate) with the gluthathione sepharose matrix and GST-AmCaM (1 hour at 4°C shaking) prior loading the mixtures into a purification column; neither His-AmCaM-like nor His-AmNC eluted together with GST-AmCaM from the glutathione sepharose matrix in either treatment (data not shown). These results suggest that the binding of the GST fraction (~25 KDa) of the GST-AmCaM fusion protein (~42 KDa) to the glutathione sepharose matrix during the incubation period is likely to destabilizes the GST-AmCaM / His-tagged *Acropora* protein complexes and disables the interactions observed using the Ni-NTA resin matrix.

To confirm the results obtained by affinity chromatography both *Acropora* His-tagged proteins were incubated (separate) with GST-AmCaM in the presence of an agarose-conjugated human CaMI antibody (1 hour at 4°C shaking). Western blots demonstrate that human anti-CaMI is specific for AmCaM as neither GST nor His-tagged AmCaM-like/AmNC were detected in the blots (Fig 4A). Despite a series of washes carried out on the agarose-conjugated complexes (3X), His-AmNC co-precipitated with GST-AmCaM and the agarose-conjugated CaMI antibody in both treatments (Fig 4B). Contrasting with these results, the CaMI antibody failed to bind to GST-AmCaM in both treatments during the His-AmCaM-like inmunoprecipitation experiments. Neither GST-CaM nor His-AmCaM-like fusion proteins were present on the pellets with the agarose-conjugated antibody but were observed together in the washes (Fig 4B-C).

4. 5. DISCUSSION

4. 5. 1. *Acropora millepora*'s EF-hand proteins.

The EF-hand family of proteins is the most studied group of intracellular molecules that bind calcium either to translate its signal or to buffer its cytosolic concentration (Carafoli, 2002; Bahler and Rhoads, 2002). EF-hand proteins bind calcium with high affinity via a helix loop helix motif (the EF-hand), which can be present between 2 to 12 times depending on the protein and its function (Haeseleer *et al.,* 2002; Carafoli, 2003).

While four EF-hand domains were identified in the AmCaM related molecules, only three were present in AmNC (Fig 1). Despite amino acid (AA) substitutions within the EF-hand domains, all EF-hand motifs present in the *Acropora* proteins were ~13 AA long. This variation in EF-hand AA composition might be responsible for the ability and affinity of the motifs to bind calcium. The residue located in the 9th position of the EF-hand loop can affect calcium-binding affinity up to 1000 fold (Burgoyne and Clague, 2003).

This is consistent with the "inactive" status designated to EF-hands I, III and IV in AmCaM-like and EF-hand III in AmNC. Protein alignments of both AmCaM-like and

AmNC against AmCaM showed AA substitutions and a high degree of dissimilarity between the "inactive" domains versus the putative "active" AmCaM EF-hand motifs. On the other hand, "active" EF-hands in AmCaM-like and AmNC showed a high degree of AA conservation with those of AmCaM (Fig 1D). Calcium-binding assays are necessary in order to confirm the predictions from sequences analyses and to characterize the calcium affinity of these *A. millepora* calcium sensors.

4. 5. 2. *Acropora* Calmodulins: AmCaM and AmCaM-like.

Primary structure analysis of both AmCaM and AmCaM-like revealed that similar to most EF-hand proteins, two calcium-binding motifs are close together forming N-terminal and C-terminal EF-hand pairs. This structural organization is believed to be responsible for the ability of some calcium sensors to interact with target proteins as a whole or as independent N-terminal or C-terminal subunits (Vetter and Leclerc, 2003). Furthermore, this structural organization might resemble the organization of an ancestral CaM molecule.

According to Baba *et al.,* (1984), modern CaM molecules evolved in the stem of eukaryotes from an ancestral CaM gene that was conserved and evolved at a slower rate than the subsequent gene duplication products due to its ability to regulate vital and multiple signalling pathways. Molecules originating from duplication of this ancestral gene evolved faster and thus established specific calcium mediated functions, which allowed for the diversification of calcium binding proteins and calcium dependent cellular activity among eukaryotes (Haeseleer *et al.,* 2002).

The discovery of a novel cDNA encoding an EF-hand named CnidEF in the sea anemone *Anthopleura elegantissima* (Hauck *et al.,* 2007) and a large variety of CaM-like coding genes in the vertebrate retina (Haeseleer *et al.,* 2002) supports these ideas and suggests common ancestry for the EF-hand family of proteins.

4. 5. 3. *Acropora* Neurocalcin: AmNC.

Neurocalcins belong to the Neuro Calcium Sensor (NCS) family of proteins, a group of molecules that regulate a variety of calcium dependent pathways (Burgoyne, 2007).

The main difference between NCSs and CaMs is a central, flexible linker region present only in CaM molecules that allows the independent interaction of both N and C terminals with target proteins. This observation is consistent with the primary structure of both AmCaM and AmNC. While AmCaM and AmCaM-like possess a central linker region of ~23-24 AAs, AmNC lacks this region (Fig 1). This observation suggests that comparable to other members of the NCS family, AmNC functions as a whole unit (Haeseleer *et al.,* 2002).

Similar to its orthologs, a putative myristoylation site that displayed the canonical MGK motif was identified at the N-terminal end of the AmNC protein. Myristoyl tails are acyl chains of 14 carbons commonly present in NCS proteins. This fatty acid post-translational modification is mainly used as a calcium-dependent membrane anchor. (O'callaghan and Burgoyne, 2003). Vertebrate Neurocalcins belong to the class NCS-B, a group of molecules that expose their myristoyl group upon calcium binding. This topological change known as the "calcium myristoyl switch" (MSM) (Burgoyne and Weiss, 2001; Burgoyne, 2007) directs them to subcellular compartments where they interact reversibly with their targets in a calcium-dependent manner.

In most molecules displaying the MSM, the first N-terminal EF-hand is inactive and functions as a hydrophobic pocket that interacts with the myristoyl group in the absence of calcium (O'callaghan and Burgoyne, 2003; Burgoyne *et al.,* 2004). The finding of only one "active" EF-hand motif in the N-terminus of the AmNC protein, together with the observation of a N-terminal region with high variation in AA composition aligning with the first EF-hand motif in the AmCaMs (Fig 1D), suggest that similar to its vertebrates counterparts, AmNC is likely to experience the MSM. Results from the Nickel affinity chromatography experiments are consistent with the theory presented above. In the absence of calcium, His-AmNC bound weakly to the Nickel affinity column (Fig 3C) thus removed with the series of washes prior elution. This suggests that under these conditions topological changes that resemble the MSM of NCS-B type proteins (Burgoyne *et al.,* 2004) might be responsible for the sequestration of the N-terminal His-tag and thus weak binding to the column.

4. 5. 4. AmCaM mediated signalling transduction mechanisms: target specificity and availability.

CaM is s a highly soluble acidic intracellular calcium-binding molecule conserved in all eukaryotes (Benain and Villalobo, 2002). Due to its role as a calcium sensor that regulates essential cellular processes such as cell cycle and calcium homeostasis its sequence is more that 90% identical (Cyert, 2001; Carafoli, 2005) among animals, plants, fungi and protozoans (Vetter and Leclerc, 2003; Haeseleer *et al.,* 2002).

In addition, CaM is considered the most versatile EF-hand calcium sensor as it regulates cytoskeleton activity, cellular metabolism, cell differentiation, proliferation, apoptosis, and metamorphosis (Benain and Villalobo, 2002; Klee and Means, 2002); and a diversity of calcium dependent secretory pathways (Dolman and Tepikin, 2006). These cellular processes are likely to play important roles during coral metamorphosis as well as during transport and secretion of organic matrix precursors necessary for skeleton deposition (Puverel *et al.,* 2005; Allemand *et al.,* 1998; Clode and Marshal, 2004).

CaM is able to interact with target molecules in both the presence and absence of calcium ions, which allows the regulation of different cellular processes in a calcium dependent and independent manner (Burgoyne and Clague, 2003). The calcium free state of CaM is known as Apo-CaM (Cyert, 2001; Haeseleer *et al.,* 2002). Apo-CaM interactions occur at low or basal cytosolic calcium levels and in some cases Apo-CaM is pre-bound to targets molecules modifying them as soon as calcium concentration rises (Bahler and Rhoads, 2001; Burgoyne and Clague, 2003). However, most of CaM interactions occur when intracellular calcium concentration reaches high μM levels (Burgoyne and Clague, 2003).

Calcium binding to CaM generates a reorganization of the helix loop helix EF-hand motifs and causes a topological change that will expose previously hidden hydrophobic residues (Ishida *et al.,* 2002). These hydrophobic amino acids in particular Methionine (Met) residues are responsible for the interaction with target molecules and recognition of CaM binding sites (Ishida *et al.,* 2002; Vetter and Leclerc, 2003). CaM regulated molecules need CaM to alter their structure and enhance/inhibit their activity in a calcium dependent manner (Vetter and Leclerc, 2003).

Despite the fact that CaM's sequence is highly conserved and identical among distant phylogenetic groups, CaM-binding regions in target proteins are very diverse. This indicates independent target evolution and diversification of calcium dependent CaM-mediated signalling pathways (Bahler and Rhoads, 2001).

4. 5. 5. AmCaM mediated signal transduction during settlement and metamorphosis.

The AmCaM temporal expression profile suggests stage specific AmCaM mediated signalling pathways, which are likely to regulate stage specific developmental processes. This could be achieved by changes on the multiple variables that control the CaM mediated translation of the calcium signal such as cytosolic calcium concentration, type of calcium oscillations, availability of target molecules as well as CaM phosphorylation profiles. It has been shown that CaM is phosphorylated *in vivo* and *in vitro* by multiple kinases. This has physiological important consequences as diverse phosphorylated CaM species have different functions (Benain and Villalobo, 2002).

Since CaM is a key molecule able to control cell differentiation, apoptosis and a diversity of calcium dependent secretory pathways (Klee and Means, 2002; Dolman and Tepikin, 2006), we postulate that CaM might regulate 1) morphogenesis during *Acropora* metamorphosis (Clode and Marshall, 2004), 2) transport of organic matrix precursors and 3) transport of calcium ions during late stages of larval development and early calcification. There is evidence demonstrating that coral CaM interacts with a calcium ATPase and that this interaction might be relevant during skeleton deposition (Zoccola el al., 2004). The identification of CaM specific targets during metamorphosis and early stages of mineralization would contribute to the identification of the CaM-mediated cellular pathways underlying morphogenesis and skeletogenesis in scleractinian corals.

4. 5. 6. *In vitro* AmCaM interactions: AmNC and AmCaM-like putative AmCaM targets during settlement and metamorphosis.

Protein-protein interaction experiments demonstrated that at least under *in vitro* conditions GST-AmCaM is able to bind both His-AmNC in a calcium dependent and independent manner (Figs 3C-4B) and His-AmCaM-like in a calcium dependent manner (Fig 3B). These observations together with the temporal expression profile of the three *Acropora* EF-hand proteins postulate AmNC and AmCaM-like as putative AmCaM targets during settlement and metamorphosis.

The lack of AmCaM-like orthologs among the available cnidarian or vertebrate databases suggests that AmCaM-like might be a coral specific gene and that its function according with its temporal expression profile might be related to coral specific processes such as metamorphosis and skeleton deposition. These observations are consistent with the idea that diversification of CaM-like molecules from a CaM ancestral gene increased the number of calcium dependent cellular processes establishing specific calcium mediated functions and pathways (Baba *et al.,* 1984; Haeseleer *et al.,* 2002).

Although functional analogy with vertebrate NC orthologs indicates a role for AmNC in vesicle transport, as they are known to interact with cytoskeleton proteins as well as with the Clathrin heavy chain, which is a major constituent of some vesicles coats (Ivings *et al.,* 2002; Haynes *et al.,* 2006); there is evidence demonstrating that Neurocalcin interacts with mRNA and the cytoskeleton and is able to control intracellular levels of cGMP in a calcium dependent manner (Burgoyne and Weiss, 2001). Functional studies are necessary to understand whether these AmCaM interactions occurs *in vivo* and represent specific AmCaM mediated transduction mechanisms regulating key developmental processes such as metamorphosis and larval skeleton deposition.

The fact that during immunoprecipitation neither AmCaM nor AmCaM-like co-precipitated with the agarose conjugated CaM antibody but were found together in the second wash (Fig 4C); suggest that the nature of the interaction between GST-AmCaM and His-AmCam-like hide AmCaM's epitopes necessary for the recognition and subsequent binding by the human CaM antibody. This result is consistent with the CaM "wrap-around" model in which cooperative folding between the N and C-

terminal domains are necessary for CaM interaction with target molecules (Haeseleer *et al.,* 2002; Carafoli, 2004).

The fact that the putative CaM-BS in both AmNC and AmCaM-like is located in between putative calcium binding sites in the target proteins (Fig 1B-C) suggests a cooperative coordination between AmCaM and EF-hands motifs in the putative target proteins. There is evidence demonstrating that in some cases CaM coordination is necessary to bind calcium or to induce exposure of hydrophobic residues in areas of inactivated calcium binding EF-hands (Ishida *et al.,* 2002; Haeseleer *et al.,* 2002).

4. 6. CHAPTER FIGURES.

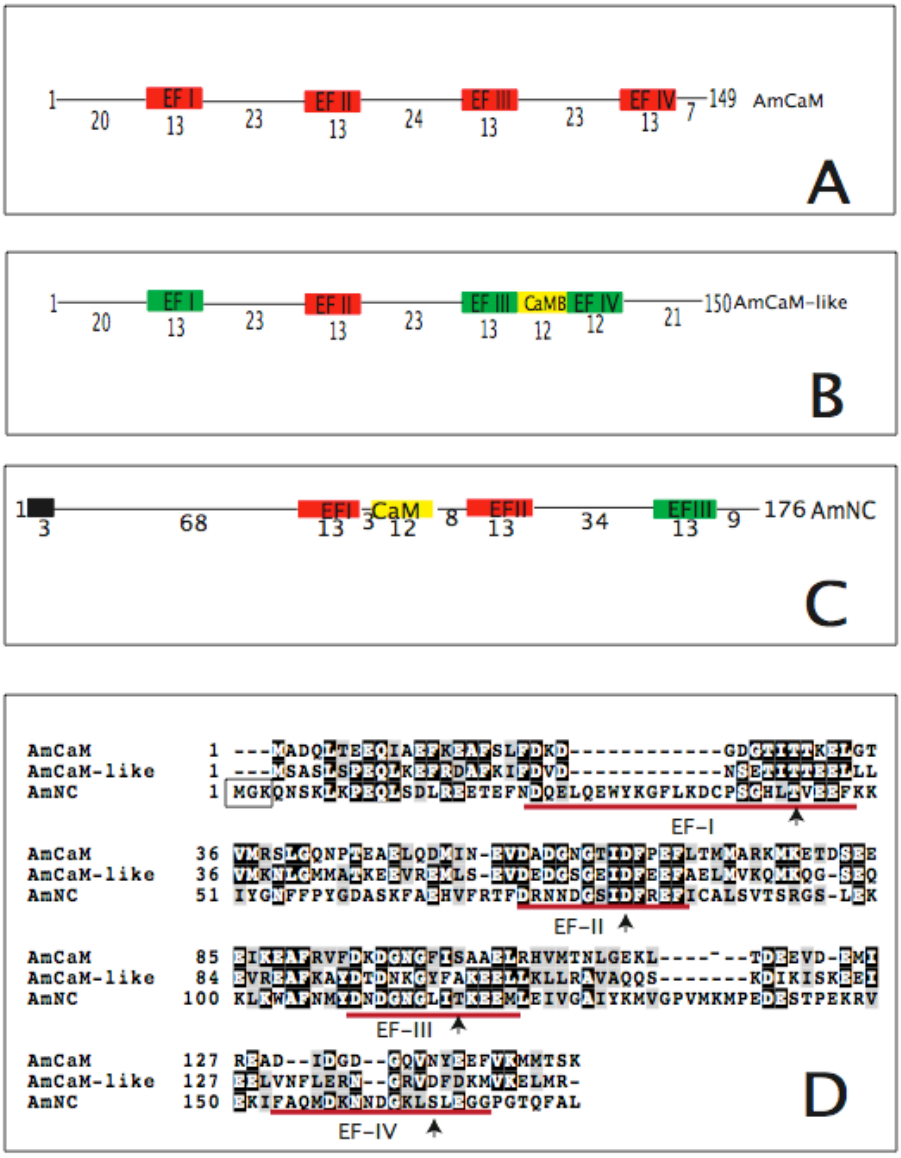

Figure 4. 1. AmEF-hand proteins: Primary structure.
Several cDNA's encoding EF-hand proteins were identified in the *A. millepora* EST data set. Two proteins belonging to the CaM family included an *Acropora* CaM ortholog (A) and one CaM-like protein likely to be coral specific were identified (B). An *Acropora* Neurocalcin ortholog that belongs to the NCS family of proteins was also found, this molecule contained a putative N-terminal myristoylation sites (C). All proteins contain EF-hand motifs. Active EF-hands (red boxes/lines). Inactive EF-hands (green boxes). Putative CaM-binding site (yellow boxes). Putative myristoylation site (black box).Numbers represent AA length. 9th position within EF-hand domains (Black arrow).

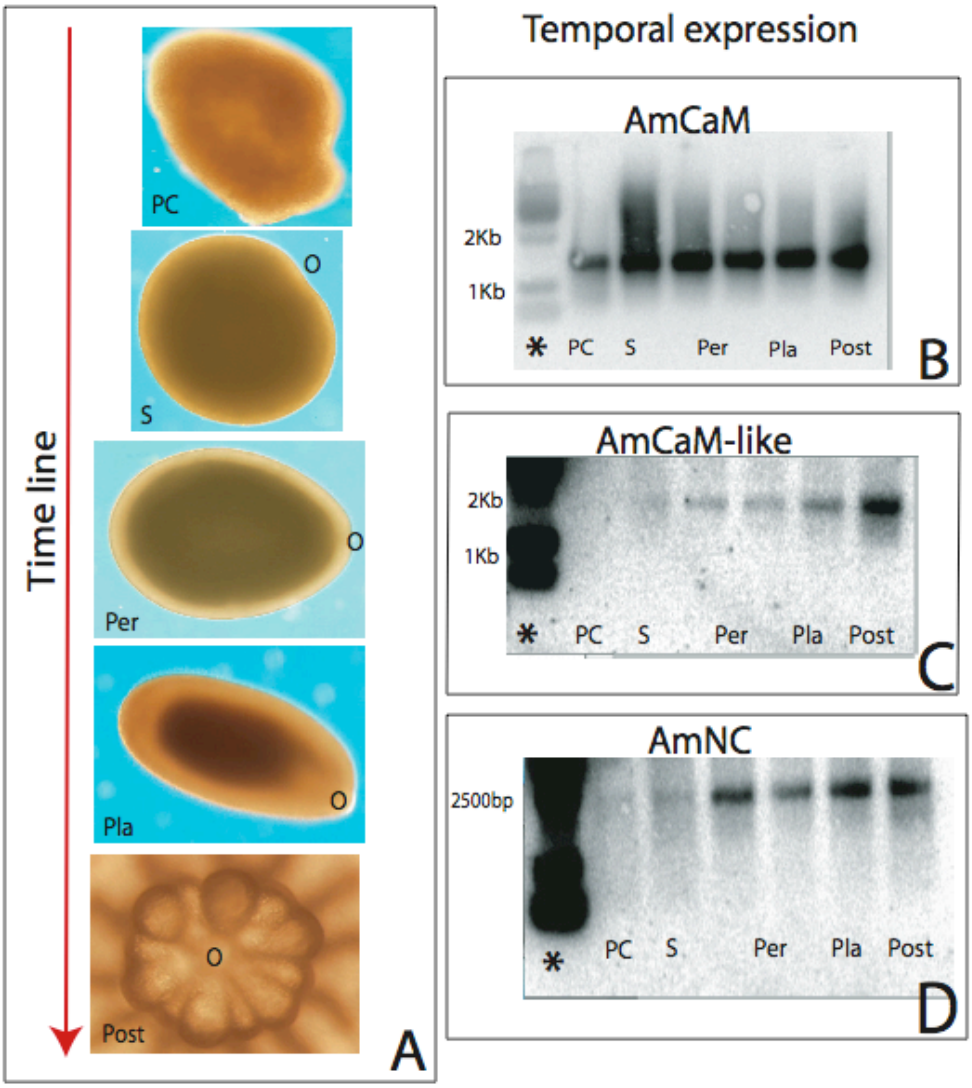

Figure 4. 2. Temporal expression of EF-hand proteins.
Following fertilization *Acropora* embryos develop into a modified blastula know as prawnchip (PC). After gastrulation (S) the two germ layers present in cnidarian (i.e endoderm and mesoderm) and the oral pore (O) are formed. A progressive oral-aboral axis elongation follows until embryos acquire a pear like shape (Per). By the time coral larva acquire the typical planula (Pla) morphology it is ready to settle and metamorphose into a calcifying, sessile polyp (Post) upon finding of exogenous settlement clues **(A).** AmCaM is expressed consistently during development, metamorphosis and post settlement stages (PC-Post)**(B).** AmCaM-like is low expressed during axis elongation stages (Per,-Pla) reaching its peak after settlement (Post)(**C).** AmNC is expressed during all axis elongation and post-settlement stages (Per to Post)**(D).** Asterisk (DNA markers).

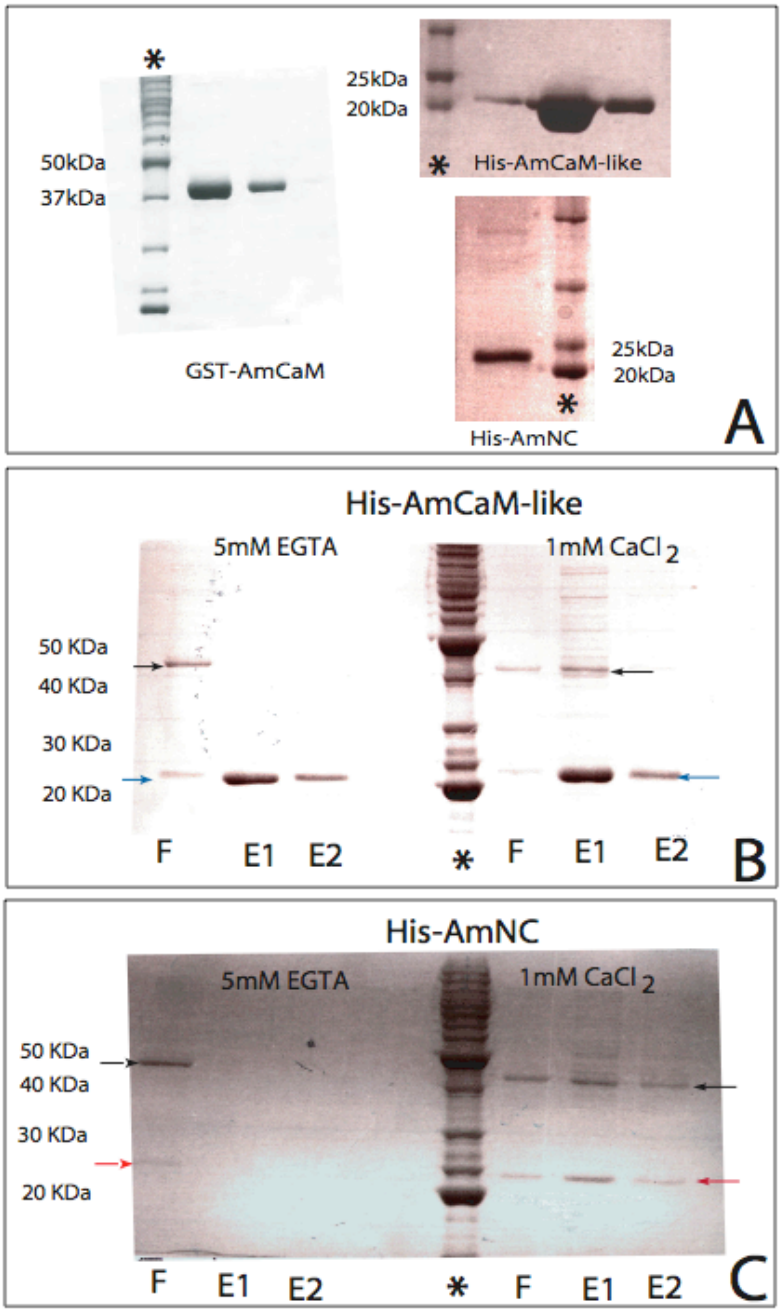

Figure 4. 3. AmCaM interacts *in vitro* with AmCaM-like and AmCaM proteins. To test for interactions with GST-AmCaM, both AmCaM-like and AmNC were expressed as His-tagged fusion proteins **(A).** GST-AmCaM (black arrow) eluted together with His-AmCaM-like (blue arrow) **(B)** and and HisAmNC (red arrow) **(C)** only in the presence of calcium during affinity chromatography experiments. Flow through (F), Elution 1 (E1), Elution 2 (E2). Protein markers (Asterisks).

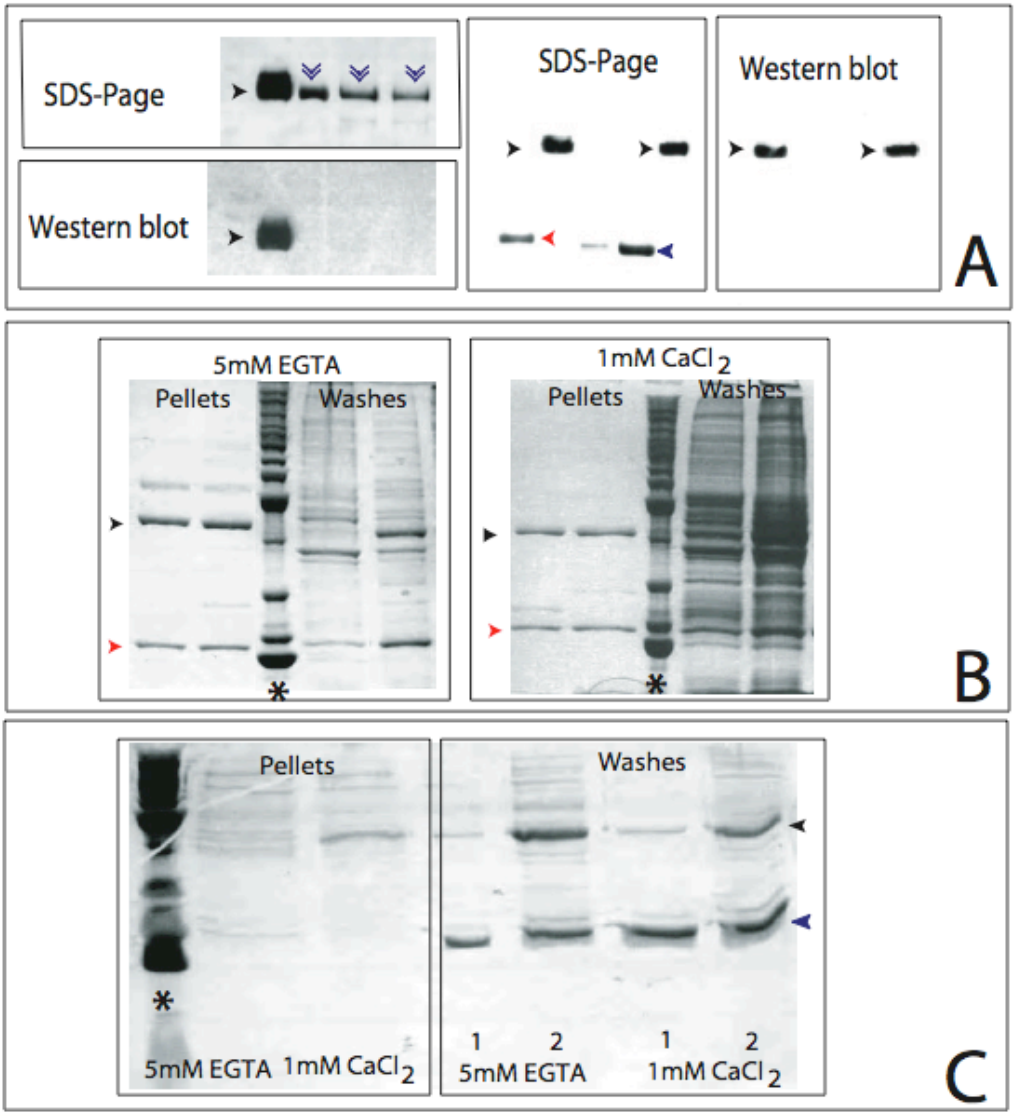

Figure 4. 4. AmCaM co-precipitates with AmCaM-like and AmCaM.
Anti human polyclonal CaMI (FL-149) antibody is specific for AmCaM (black arrow) as neither GST-AmCaM-like (double blue arrow) nor His-AmCaM-like (red arrow)/His- AmNC (blue arrow) proteins were detected by western blots **(A).** His-AmNC (red arrow) co-precipitated with GST-AmCaM in both the presence and the absence of calcium **(B).** Neither GST-AmCaM nor His-CaM-likeB co-precipitated with the Anti human CaM antibody in the prescence or absence of calcium. Both proteins were present together in the second wash **(C).** First wash (1). Second wash (2). Protein markers (Asterisks).

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CHAPTER 5. cDNA microarray analysis of metamorphosis and the early stages of calcification in the scleractinian coral *Acropora millepora.*

5.1. ABSTRACT.

To elucidate the molecular mechanisms underlying metamorphosis and the early stages of calcification in the complex scleractinian coral *Acropora millepora,* gene expression cDNA microarray analysis was performed between aposymbiotic planulae, flat and primary polyps as well as symbiotic adult tips. The most highly up-regulated genes in planulae are involved in calcium signalling, lipid metabolism and developmentally regulated signalling pathways. Transcripts up-regulated in post-settlement juvenile stages are predominantly implicated in tissue morphogenesis, cell division and responses to oxidative stress, whereas transcripts that showed the highest expression in adult polyps are likely to have roles in responses to abiotic stimuli, asexual reproduction and growth. Transcripts up-regulated in all settled stages included carbonic anhydrases and molecules involved in O-glycosylation and organic matrix synthesis, thus presumably reflecting roles in skeleton deposition. Additionally, our results suggest that an overlap of intracellular secondary messengers that mediate metamorphosis in *Acropora* is likely to occur between planulae and settled juvenile polyps. While calcium and lipid signalling might play important roles in the regulation of cellular activity at the planula stage, ROS signalling seems to be a key regulator during morphogenesis of adult structures. Finally, our results are consistent with the idea that differential expression of organic matrix components is responsible for the two types of calcification observed in corals 1) basal plate formation and 2) the fiber-like calcification characteristic of adult polyps.

5. 2. INTRODUCTION.

As in the case of many marine invertebrates, the larvae of scleractinian corals undergo metamorphosis - a dramatic morphological transformation during the settlement process. At this time, planula larvae transform from non-calcifying, demersal, motile organisms into sessile, calcifying, benthic juvenile polyps. Metamorphic morphogenesis in scleractinian corals is a distinctive process that differs from the gradual metamorphosis experienced by the closely related anthozoan *N. vectensis*

(Hand and Uhlinger, 1992). A unique change during metamorphosis of coral planulae involves the reorganization of the aboral ectoderm from a columnar epithelium into a squamous calcifying tissue known as the calicoblastic ectoderm (Vandermeulen, 1974; Clode and Marshall, 2004). This newly formed cell population secretes an organic matrix able to induce and control skeleton deposition (Puverel *et al.,* 2005) allowing the formation of coral reefs.

Scleractinian corals play an important ecological role, as their skeletons constitute the framework for coral reefs, one of the most productive ecosystems on earth (Gunderson, 2007). Despite this, very little is known regarding the molecular mechanisms and gene networks underlying metamorphic morphogenesis and skeleton deposition in reef-building corals. Gene expression microarray analyses represent a powerful tool to characterize such gene networks due to their ability to examine transcription levels for a high number of genes simultaneously. This approach has been used to characterize gene expression profiles during key transitions in the life cycle of a diverse range of organisms (Hill et al. 2000; Arbeitman et al. 2002; Baldessari et al. 2005; Wei et al. 2006; Azumi et al. 2007).

Regarding marine invertebrates, Williams et al. (2009) studied the gene expression changes underlying settlement and metamorphosis in the abalone *Haliotis asinine* using a cDNA platform. Although several groups have described metamorphosis in scyphozoans (Yuan et al. 2008; Nakanishi et al. 2008), hydroids (Katsukura et al. 2003) and non-calcifying anthozoans (Hand and Uhlinger, 1992), there have been few microarray analyses of the process. Recently, Grasso et al. (2008) compared gene expression profiles between four key stages during the development of *Acropora millepora* and Reyes-Bermudez et al., (in press) compared changes in the transcriptome between planulae, primary and adult polyps during settlement and metamorphosis in the Caribbean robust coral *Montastraea faveolata*.

In both cases the cDNA platforms represented only a fraction of the transcriptome - 5081 and 1310 unigenes for *Acropora* and *Montastraea* respectively- thus limiting the scope of the approach. In this study we compare changes in the transcriptome between *Acropora millepora* planulae, flat, primary and adult polyps using a new

generation cDNA array representing 8606 unigenes. Furthermore, the fact that two consecutive juvenile stages- flat and primary polyps- were selected for this study will increase the resolution of the analysis focusing of the gene networks underling settlement and memamorphosis as well as complementing previous analyses.

Microarray analysis during late stages of coral development will allow the identification of convergent and/or ancestral molecular pathways regulating morphogenetic changes not only among reef-building corals but also across the Cnidaria. Furthermore, studies concerning the molecular basis of coral calcification are necessary as they are likely to provide information valuable for the management and conservation of coral reefs.

5. 3. MATERIAL AND METHODS.

5. 3. 1. Collection of samples

Acropora millepora mature colonies were collected during the 2005-spawning event at Magnetic Island (Lat: 19° 7' 60 S, Long: 146° 49' 60 E, Queensland, Australia). Following spawning, gametes from several colonies were mixed to facilitate fertilisation, and the resulting embryos maintained in containers containing fresh seawater for 2h. Embryos were maintained in 1μm filtered seawater, with water changes several times per day, until they reached the planula stage. At this point, a subset of planula larvae was collected and preserved in liquid nitrogen. Remaining larvae were transferred to 190mm petri dishes and allowed to metamorphose and settle.

After 2 days, a subset of settled larvae that had totally flattened down along the oral-aboral axis were collected and preserved in liquid nitrogen. The remaining settled larvae were kept and allowed to further develop until they reached the characteristic primary polyp morphology; at this stage samples were collected and transferred to liquid nitrogen. Adult tips were obtained by braking off small fragments from the collected colonies selected for spawning. Fragments were frozen in liquid nitrogen. All samples were kept in liquid nitrogen prior transport to ANU for posterior RNA extraction and microarray hybridizations.

5. 3. 2. RNA extraction and amplification

Total RNA from frozen coral tissues was isolated using Qiazol lysis reagent (Qiagen) following product specifications. Both adult and larval tissues were homogenized using a pre-chilled mortar and pestle. Frozen coral powder was transferred directly to Qiazol. Two chloroform extractions were performed, followed by isopropanol precipitation and two washes in 80% ethanol. RNA pellets were re-dissolved in nuclease-free water. Due to the insufficiency of planula and polyp RNA, all RNA samples were amplified using the MessageAmp II aRNA kit (Ambion) with 1ug of total RNA as input.

5. 3. 4. Microarray hybridisation and array description.

The array used in this experiment consisted of 16.000 spots representing 8606 unigenes. Array description and specification have been previously described in Bay et al., (2009). All of the material used for making the libraries came from Nelly Bay, Magnetic Island, Queensland, Australia (19°08'S 146°50'E). Generation and spotting of cDNAs as well as EST analyses are previously described in (Grasso *et al.,* 2008).

Prior to hybridization, microarrays were post-processed by: 1) UV crosslinking at 60 mJ; 2) a "shampoo" treatment (3x SSC, 0.2% SDS at 65^{o}C); 3) blocking with 5.5g succinic anhydride dissolved in 335mL 1-methyl-2-pyrrilidinone and 15mL sodium borate; and 4) drying via centrifugation. Five micrograms of aRNA per sample were primed with 5ug/uL random nonamer for 10 min at 70^{o}C. Reverse transcription (RT) lasted for 2 hr at 50^{o}C using a master mix containing a 4:1 ratio of aminoallyl-dUTP to TTP. Following RT, single-stranded RNA was hydrolyzed by incubating the RT reactions in 10 uL 0.5 M EDTA and 10 uL 1 M NaOH for 15 min at 65^{o}C. After hydrolysis, RT reactions were cleaned using MinElute Reaction Purification columns (Qiagen). Cy3 and Cy5 dyes (GE Healthcare) were dissolved in 12 uL DMSO, and the coupling reactions lasted for 2 hr at room temperature in the dark. Dye-coupled cDNAs were cleaned (MinElute columns), and appropriate Cy3 and Cy5 labeled cDNAs were mixed together in a hybridization buffer containing 0.25 % SDS, 25 mM HEPES, and 3x SSC. The hybridization mixtures were boiled for 2 min at 99^{o}C then

allowed to cool at room temperature for 5 min. The cooled hybridization mixtures were pipetted to the printed arrays and left overnight at 63 oC. Microarrays were washed twice in 0.6x SSC and 0.01 % SDS followed by a rinse in 0.06x SSC and dried via centrifugation. Slides were immediately scanned using a GenePix 4200A scanner.

5. 3. 5. Experimental design and microarray data analysis

To understand the molecular mechanisms underlying skeleton deposition in *A. millepora* gene expression was compared between 1) aposymbiotic non-calcifying planulae, 2) aposymbiotic calcifying flat polyp, 3) aposymbiotic calcifying primary polyp and 4) symbiotic adult tips. The experiment followed a loop design in which all life stages were directly compared against each other. Hybridizations were performed in duplicate (n=2 for each developmental stage and included dye swapping between technical replicates (Fig 5.1).

While pre-processing of microarray intensity data was performed using Spot http://www.csiro.au/products/ps1ry.html all further analyses were carried out using the limma package (Smyth, 2005) for the R system http://www.r-project.org/. Print-tip loess normalisation (Smyth and Speed, 2003) was performed on each slide. Quantile normalisation was applied to mean log-intensities in order to make the distributions essentially the same across arrays. The methodology used for statistical analysis is described in Smyth (2004). The prior probability of differential expression, for each pair of comparisons between stages, was taken as 0.1. The Benjamini and Hochberg method (Benjamini and Hochberg, 1991) was used to adjust the sequence-wise p-values, so that a choice of sequences for which the adjusted p-value is at most 0.01 identifies a set of differentially expressed genes in which 1% may be falsely identified as differentially expressed.

To visualize the temporal expression of DEGs, K-means clustering was performed using the TIGR TMEV 4.0 package within the TM4 software suite. To assess over-representation of Gene Ontology (GO) terms in the lists of significant genes, default values in GOEAST (Zheng and Wang 2008) were used and GO categories selected based on p-values ($p = <0.05$). GOEAST identifies significantly enriched GO terms

among a list of genes by calculating the hypergeometric probability that a particular GO term was represented in a given set of DEGs by more microarray features than would be expected by chance. We viewed GOEAST results with caution and only focused on over-represented categories that contained two or more genes.

5. 4. RESULTS

To better understand the molecular mechanisms regulating the early stages of calcification in the scleractinian coral *Acropora millepora,* cDNA microarray analysis was used to compare changes in the transcriptome between planula (Pla), flat polyp (F), primary polyp (Po) and symbiotic adult tips (T) (Fig 5.1). From the 8606 unigenes printed on the array, a total of 1189 were identified as differentially expressed genes (DEGs) between any two stages ($p = <0.01$). While 52% of DEGs had annotated hits according to BLASTx (E-value $\leq 1x\ 10^{-5}$) the remaining 48% did not have significant database matches.

DEGs were clustered in four groups that represented major expression profiles. DEGs were further grouped into cluster-specific expression patterns (EPs) within each cluster depending on the common stages in which they were up or down-regulated. Although some EPs from different clusters displayed similar overall expression patterns (i.e. up or downregulated in similar stages) the expression levels (values) for DEGs within those groups differed drastically from one another thus establishing independent and distinct gene expression profiles. While table 5.1 summarized data for all clusters and their expression patterns, table 5.2 summarized the functional annotations, clone ID and expression data for all DEGs mentioned below. Table S1 contains expression values for all DEGs and table S2 summarized the functional annotations (GO and KEGG pathways) for all DEGs with significant BLASTx hits.

5. 4. 1. Cluster I. Genes down-regulated after settlement: highest expression in planulae.

Cluster I (CI) consists of 547 unigenes (46% of DEGs) from which 284 were annotated and represented 45% of all annotated sequences. Absolute fold changes

(FCs) in CI were as high as ~10-fold. CI DEGs were distributed in 8 distinct EPs named here CI-EP1 to CI-EP8 (Fig 5.2).

CI-EP1 contains 158 unigenes that were down-regulated up to ~10-fold in F, Po and T in comparison to Pla. CI-EP-2 consists of 16 unigenes that were down-regulated up to ~ 4-fold in F and T but remained stable in Po when compare to Pla. CI-EP3 has 137 DEGs down-regulated up to ~ 4-fold in T but stable in F and Po relative to Pla. CI-EP4 contains only 2 genes that were up-regulated up to ~ 2-fold in F but were down-regulated up to ~ 3-fold in Po and T when compared to Pla. CI-EP5 groups 87 unigenes that were down-regulated up to ~ 4-fold in Po and T but remained stable in F when compared to Pla. CI-EP6 contains 41 genes that were down-regulated up to ~ 4-fold in F and Po but were stable in T relative to Pla. CI-EP7 has 77 genes that were only down-regulated up to ~ 3-fold in Po but were stable in F and T when compare to Pla. Finally, CI-EP8 contains 29 genes that were down-regulated up to ~ 3-fold in F but remained stable in Po and T in relation to Pla (Fig 5.2).

Statistically over-represented GO categories in CI include: bioluminescence (GO:0008218) in CI-EP1, positive regulation of apoptosis (GO:0043065) in CI-EP15-7, larval development (GO:0002164) in CI-EP6, identical protein binding (GO:0042802) in CI-EP7, regulation of transport (GO:0051049) in CI-EP8 and phospholipid biosynthetic process (GO:0008654) in CI-EP6. This last category is consistent with the down-regulation after settlement of 16 DEGs that mapped to KEGG pathways involved in lipid metabolism (Table 5.2). Although lipid metabolisms seems to be the main energy source at the planula stage, dowregulation after settlement of a number of genes involved in carbohydrate metabolisms (PATH: ko00051, PATH: ko00010, PATH: ko00030) indicates that lipids are not the only source of energy for planula larvae.

Consistently with the over-representation of GO category GO:0051049 (regulation of transport), the identification of four genes involved in the regulation of the actin cytoskeleton (PATH: ko04810) that were down-regulated after settlement suggests active intracellular transport in planulae tissues. This is consistent with down-regulation after settlement of coral orthologs of calumenin (C_mge-C006-A6-

pre40_T3 and C_mge-A044-E12-post92) and calreticulin (C_MGE-A022-E1-post4-T3) known to regulate transport from the ER and signal transduction in vertebrates (Honore and Vorum, 2000; Jung *et al.,* 2006; Vallar *et al.,* 1999; Tran *et al.,* 2002). Down-regulation after settlement of three DEGs encoding proteins involved in the neuroactive ligand-receptor interaction pathway (PATH: ko04080) suggests that these molecules might be involved in the transduction of external stimuli into cellular activity during settlement and metamorphosis.

In a similar way, down-regulation after settlement of components of the TGF-beta (PATH: ko04350), Notch (PATH: ko04330) and MAPK (PATH: ko04010), signaling pathways (Table 5.2), which are conserved mechanisms able to regulate apoptosis, cell differentiation, proliferation and migrations across metazoa (Gerhart, 1999) suggests that settlement and metamorphosis in *A. millepora* is regulated by conserved signal transduction mechanisms. Furthermore, down-regulation in postsettlement stages of a number of DEGs encoding calcium-binding proteins from the EF-hand family indicates that calcium signalling plays and important role during metamorphosis and settlement in *A. millepora*. Finally, down-regulation after settlement of an Aristaless (S_mge-C015-G2-pre14_T3) and a number of bone morphogenetic protein (S_MGE-A049-C6-post42-T3, C_mge-B022-H10-prawn79 and S_MGE-B025-B6-prawn41_T3) orthologs suggests that these molecules might establish morphogenetic borders in *Acropora* planulae as they do during development in other better characterized biological systems.

5. 4. 2. Cluster II. Genes up-regulated after settlement: highest expression in calcifying stages.

Cluster II (CII) contains 84 unigenes (7% of DEGs) from which 38 were annotated and represented 6% of all annotated sequences. Absolute FCs in CII were as high as ~14-fold. CII DEGs were distributed in 4 distinct expression patterns termed CII-EP1 to CII-EP4 (Fig 5.3).

CII-EP1 contains only 1 unigene (S_MGE-A023-D8) encoding a major yolk protein ortholog that was stable in F and up-regulated up to ~9-fold in Po and T in comparison to Pla. CII-EP-2 groups 52 unigenes that were up-regulated up to ~ 14-

fold in all three stages relative to Pla. CII-EP3 has 2 non-annotated DEGs that were up-regulated up to ~ 7-fold in F and T but up-regulated up to ~ 2 fold in T when compare to Pla. CII-EP4 contains 29 DEGs that were up-regulated up to ~ 12-fold in F and Po but remained stable in T relative to Pla.

Statistically over-represented GO categories in CII include: hydrolyzing O-glycosyl compounds (GO:0004553), carbohydrate binding (GO:0030246), and lyase activity (GO:0016829), which included two types of carbonic anhydrases and a carbonic anhydrase inhibitor in CII-EP2 and metalloendopeptidase activity (GO:0004222) in CII-EP4 (Table 5.2). All molecules belonging to the GO category GO:0004553 (hydrolyzing O-glycosyl compounds) mapped to the O-Glycan biosynthesis KEGG pathway (PATH: ko00512). These results are likely to reflect the OM synthesis and carbonate chemistry mechanisms underlying skeleton deposition. Consistently, up-regulation in CII-EP2-4 of a dynein light chain 2 (C_MGE-A027-C11-post82) and a tubulin alpha-1 chain (C_mge-A048-D1-post3-T3) orthologs indicates active intracellular transport possibly of OM precursors.

In a similar way, up-regulation of a bone morphogenetic protein ortholog (S_mge-A048-B3) that mapped to the TGF-beta signaling pathway (PATH: ko04350) as well as a dual specificity phosphatase 7 ortholog (C_D019-G3_23) which is involved in the MAPK signaling pathway (PATH: ko04010) indicates that these signal transduction mechanisms might regulate calcification-specific processes such as calicoblast differentiation and production of the OM.

5. 4. 3. Cluster III. Genes up-regulated after settlement but stable or down-regulated in adult: highest expression in juvenile settled stages.

Cluster III (CIII) contains 243 unigenes (20% of DEGs) from which 132 were annotated and represented 21% of all annotated sequences. Absolute FCs in CIII were as high as ~4-fold. CIII DEGs were distributed in 7 distinct expression patterns referred here as CIII-EP1 to CIII-EP7 (Fig 5.3).

CIII-EP1 contains 52 DEGs that were up-regulated up to ~ 4-fold only in Po and remained stable in F and T when compared to Pla. CIII-EP-2 groups 75 unigenes that

were up-regulated up to ~ 2-fold only in F and remained stable in P and T when compared to Pla. These two EPs might reflect the regulation of stage specific processes such as basal plate formation and morphogenesis of adult structures. CIII-EP3 has 27 DEGs that were up-regulated up to ~ 2-fold in F but remained stable in Po and were down-regulated up to ~ 3 fold in T relative to Pla. CIII-EP4 contains 59 DEGs that were up-regulated up to ~ 3-fold in F and Po but remained stable in T relative to Pla.

CIII-EP5 consists of 4 unigenes all up-regulated up to ~ 2-fold in all three stages relative to Pla. The only annotated gene in this EP encodes a coral ortholog of a heterogeneous nuclear ribonucleoprotein a-b (C_D045-F12_94). CIII-EP6 groups 12 DEGs that were up-regulated up to ~ 3-fold in F and Po and down-regulated up to ~ 4-fold in T when compared to Pla. Finally, CIII-EP7 contains 14 DEGs that were stable in F, up-regulated up to ~ 2-fold in Po and down-regulated up to ~ 3-fold in T in relation to Pla.

Statistically over-represented GO categories in CIII include: sequence-specific DNA binding (GO:0043565) in CIII-EP1, cellular carbohydrate metabolic process (GO:0044262), aerobic respiration (GO:0009060) and positive regulation of cell differentiation (GO:0045597) in CIII-EP2, oxidoreductase activity (GO:0016491) and response to oxidative stress (GO:0006979) in CIII-EP3-7 and transcription factor activity (GO:0003700), embryonic development ending in birth or egg hatching (GO:0009792), regulation of anatomical structure morphogenesis (GO:0022603), cation transport (GO:0006812) and anion transport (GO:0006820) in CIII-EP4.

These results suggest that although F and Po are distinct transcriptional stages they both express a common subset of DEGs that might regulate morphogenesis of adult structures (GO:0009792 and GO:0022603) and ion transport (GO:0006812 and GO:0006820) able to control larval skeleton deposition. Up-regulation in F (CIII-EP3) of the novel extracellular matrix protein Galaxin-like (C_MGE-A006-D10-28975-T), likely to be involved in basal plate formation together with up-regulation in Po (CIII-EP1-7) of two extracellular matrix glycoproteins: 1) Echinonectin (S_mge-A030-F6-post45-T3) and 2) E-selectin (S_MGE-A004-H2-pstH9715-T3) suggests

overlapping of extracellular matrix proteins that might differentially regulate skeleton deposition and/or cell adhesion during morphogenesis of adult structures. Furthermore, up-regulation in F (CIII-EP2) of bone morphogenetic protein type 1b (S_MGE-A023-C10-post74-T3) and a nemo-like kinase (C_mge-B027-F10-prawn77) suggests that similar to *Drosophila* sp an antagonic mechanisms involving nemo kinase regulation of BMP signalling (Zeng et al., 2007), is mediating cell differentiation during metamorphosis in *Acropora.*

Up-regulation in CIII-EP2 of two unigenes (S_mge-A030-E5-post36-T3 and S_mge-A037-A6-post40-T3) which orthologs are known to regulate dorso-ventral axial formation (formin 2 and modulator of activity of ets genes) (PATH: ko04320) in bilaterians suggests that cell differentiation and morphogenesis into adult structures is initiated in F. In a similar way, up regulation of two DEGs C_mge-B021-H10-prawn79 and S_MGE-A017-E2-post12-T3) in CIII-EP1, which encode orthologhs of cyclin b3 and polymerase delta 4 and mapped to cell cycle (PATH: ko041100) and DNA replication (PATH: ko03030) pathways respectively suggest that in Po cell division is actively occurring.

DEGs involved in oxidative stress response (GO:0006979) were also up-regulated in both juvenile settled stages which is consistent with the up-regulation of a number of genes involve in carbohydrate and lipid metabolic pathways able to stimulate aerobic respiration (GO:0009060) in F. These DEGs encode coral orthologs of an isocitrate lyase (C_mge-B045-B10-prawn73) that mapped to the KEGG PATH: ko00630, an aldehyde dehydrogenase a1 (S_MGE-A050-D9) that mapped to the KEGG PATH: ko00010 and an acyl-coenzyme ac-4 (C_mge-B044-C1-prawn2_T) that mapped to the KEGG PATH: ko00071.

5. 4. 4. Cluster IV. Genes stable or differentially expressed in juvenile stages but up-regulated in adult: highest expression in adult tips.

Cluster IV (CIV) contains 315 DEGs (27% of DEGs) from which 172 were annotated and represented 28% of all annotated sequences. Absolute FCs in CIV were as high as ~20-fold. CIV DEGs were distributed in 7 distinct expression patterns named here CIV-EP1 to CIV-EP7 (Fig 5.4).

CIV-EP1 contains 244 DEGs that were up-regulated up to ~ 20-fold only in T but remained stable in F and Po relative to Pla. CIV-EP2 groups 26 unigenes that were up-regulated up to ~ 9-fold in F and T but and remained stable in Po when compared to Pla. CIV-EP3 also contains 26 genes that were stable in F and up-regulated up to ~ 4-fold in Po and T in relation to Pla. CIV-EP4 has 12 genes that were up-regulated to ~ 10-fold in all three stages when compare to Pla. CIV-EP5 groups only 3 DEGs that were down-regulated up to ~ 2-fold in F and Po but up-regulated up to ~ 2-fold in T relative to planula. The only two annotated sequences in this EP encode coral orthologs of an ADAM metallopeptidase (S_MGE-A006-B9-28965-T3) and a frizzled-related protein (C_D045-C10_75).

CIV-EP6 includes only 3 genes that were down-regulated up to ~ 2-fold in F, stable in Po and up-regulated up to ~ 2-fold in T relative to Pla. All three DEGs in this group were annotated and encode *Acropora* orthologs of a calmodulin-like molecule (C_MGE-B015-F1-prawn5_T), an epididymal secretory protein e1 (C_MGE-A021-A1-post0-T3) and a n-myc downstream regulated protein (C_D003-B10). CIV-EP7 consists only of 1 non-annotated gene which expression was stable in F, down-regulated ~ 2-fold in Po and up-regulated ~ 2-fold in T relative to Pla.

Statistically over-represented GO categories in CIV include: translation (GO:0006412), pattern specification process (GO:0007389) and regulation of cell differentiation (GO:0045595) in CIV-EP1. This is consistent with the up-regulation in the same expression patter of a number of components of conserved signal transduction pathways known to regulate cell differentiation, proliferation and migration across metazoa and a number of molecules able to regulate the actin cytoskeleton. In a similar way, statistically over-represented GO categories also include: transcription regulator activity (GO:0030528) and responses to abiotic stimulus (GO:0009628) in CIV-EP2, skeletal-system development (GO:0001501), extracellular matrix production (GO:0044420) and defence response (GO:0006952) in CIV-EP3. The only statistically over-represented GO category in CIV-EP4 was anatomical structure development (GO:0048856) (Table 5.2).

Components of of conserved signal transduction pathways included: 1) a coral snail 1 ortholog (S_mge-A045-D10-post75-T3) that mapped to the KEGG pathway PATH: ko04520, 2) a beta-catenin ortholog (S_GS01vA0) that mapped to the Wnt signaling pathway (PATH: ko04310), 3) a Notch-like protein (S_MGE-A023-F2) which mapped to the Notch signaling pathway (PATH: ko04330) and 4) a heat shock protein 70 (S_MGE-A017-H8) that mapped to the MAPK signaling pathway (PATH: ko04010). The regulators of the actin cytoskeleton (PATH: ko04810) included: 1) a profilin (C_D006-B2), 2) an alpha 1 (C_mge-A043-F1-post5-T3), 3) an actin (C_MGE-A049-H4-post31-T) and 3) a Gelsolin (C_D009-C6) *Acropora* orthologs.

The extracellular matrix proteins included two Alpha-1 type collagen-coding genes (C_GS01AD12 and C_mge-A045-C3), and a coral protein containing a collagen triple helix repeat (C_MGE-A012-H12-86595-T), this molecules also mapped to the KEGG ECM-receptor interaction pathway (PATH: ko04512). Upregulation of two calmodulin-like genes in CIV-EP3 (C_mge-B027-E12-prawn92) and 6 (C_MGE-B015-F1-prawn5_T) suggest a diversification of calcium sensors that could mediate specific calcium dependent cellular pathways.

5. 5. DISCUSSION.

5. 5. 1. Differential gene expression profiles were identified between larval, juvenile and adult stages during the life history of *A. millepora*.

Gene expression microarray analysis identified four main expression profiles between the four developmental time points compared in this study. From the 8606 unigenes assayed on the chip, a total of 1189 (14%) were differentially expressed ($p = <0.01$). 52% of all DEGs (623) were annotated sequences. While 547 DEG's (46%) had the highest expression at the planula stage (CI), 315 (27%) had the highest expression in adults (CIV), 243 (20%) were highest expressed in settled juvenile stages (CIII) and the remaining 84 (7%) were up-regulated after settlement and had the highest expression in calcifying stages (CII)(Table 5.1). These results suggest that the contrasting physiological and ecological conditions experienced by *Acropora* during its life history correlates with the use of precise gene networks likely to control stage-specific responses to their environment.

While the free-swimming planula is aposymbiotic and non-calcifying, sessile juvenile polyps remain aposymbiotic but have switched to a calcifying life style and are experiencing the morphogenetic changes that will lead to the formation of adult structures. On the other hand, adult *Acropora* are colonies of symbiotic polyps supporting high levels of calcification and interacting with a diverse range of microrganisms as well as *Symbiodinium* spp., establishing the biological entity known as the coral holobiont (Miller and Bassler, 2001; Schwarz *et al.,* 2008; Siboni *et al.,* 2008; Marhaver *et al.,* 2008; Harel *et al.,* 2008). Both the ecological and physiological discrepancies observed between the different *Acropora* life stages suggest that planulae, juvenile polyps and adult tips are transcriptionally distinct stages in which changes in the transcriptome reflect responses to differing physiological and ecological conditions.

Consistent with this, Grasso *et al.,* (2008) identified stage-specific (prawnchip, planula, polyp, adult) transcription profiles during *A. millepora* development for 1084 DEGs using and array that contained 5081 unigenes. In a similar way, Reyes-Bermudez *et al.,* (in press) reported stage-specific gene expression patterns for planulae, primary polyp and adult colonies during the life cycle of the "Robust" coral *Montastraea faveolata*. Furthermore, five temporal gene expression profiles were reported during metamorphosis and settlement of the abalone *Haliotis asinine* (Williams *et al.,* 2009) and Azumi *et al.,* (2007) reported changes in the transcriptome for most *C. intestinalis* genes during its life history. The fact that 66% of all DEGs (CI and CIII) were differentially expressed in larval and juvenile stages, suggests that although some of these genes might have a physiological role it is likely that this subset of genes regulate the morphogenetic processes underlying settlement and metamorphosis in *Acropora*. This is consistent with the observation that 77% of all DEGs were differentially expressed between larval and juvenile stages during late development in *M. faveolata* (Reyes-Bermudez *et al.,* in press). Likewise, Azumi *et al.,* (2007) reported that 72% of all assayed genes were differentially expressed during development of *C. intestinalis*.

On the other hand, the 34% (399 DEGs) that showed the highest expression in adult (CIV) and calcifying stages (CII) are likely to be involved in adult-specific processes such as sexual reproduction, responses to the environment, symbiosis and skeleton

deposition. Azumi *et al.,* (2007) suggested that adult gene expression in *C. intestinalis* responds to pollutant chemicals, physiological stress and bacterial and viral infections. The fact that the percentage of DEGs with highest expression in adult stages drastically differed between this experiment (27%) and the previous *Acropora* developmental microarray study (4%) could be explained by the low proportion of adult material printed in the chip containing 5081 unigenes used by Grasso *et al.,* (2008) rather than to biological conditions.

In contrast with the results reported by Azumi *et al.,* (2007) and Reyes-Bermudez *et al.,* (in press) in which 72% and 66% of genes examined were differentially expressed at some stage during the life histories of *C. intestinalis* and *M. faveolata* respectively; the 1189 DEGs identified in this study only accounts for 14% of the total number of genes (8606) on the array. Although this number (14%) is comparable to that (21% of 5081 unigenes) reported by Grasso *et al.,* (2008), the different size of the arrays, the proportions of printed transcripts from each developmental stage and the abundance of particular transcripts in the EST libraries, complicate direct comparison of overall expression patterns. Furthermore, in all of these cases the number of unigenes on the arrays represents only a fraction of the species' transcriptome, thus complicating direct comparisons between datasets.

5. 5. 2. Planula larvae: a connection with Bilateria.

The higher proportion of annotated sequences differentially expressed in larval and juvenile stages (CI, 45% and CIII, 21%) is consistent with the results obtained for the "Robust" coral *M. faveolata*; while 45% of differentially expressed unigenes with annotation were in the planula stage and 39% in juvenile polyps, only 16% were in the adult cluster (Reyes-Bermudez *et al.,* in press). Likewise, Azumi *et al.,* (2007), reported a high number of annotated sequences among the genes that were up-regulated in early developmental stages during *C. intestinalis* life cycle and Grasso *et al.,* (2008) reported that 89% of the genes up-regulated in prawnchip had matches in the databases. However, it is also possible that the higher porortion of annotated sequences is due to the overrepresentation of developmental genes in the databases.

Recent phylogenetic analysis, suggest that both cnidarians and bilaterians evolved from a common ancestor that resembled modern planula larvae and was to some extent bilaterally symmetric (Baguna *et al.,* 2008). Furthermore, it has been proposed that similar groups of genes control morphological transitions across metazoans with biphasic life cycle (Hayland and Moroz, 2006) and there is evidence showing that developmental signalling pathways such as notch, wnt, hedgehog, and TGF-β have been conserved during metazoan evolution (Matus *et al.,* 2008; Matus *et al.,* 2007; Gordon *et al.,* 2008). According to this it is reasonable to think that coral planula and juvenile gene expression might reflect the common ancestry of developmental mechanisms.

On the other hand, the high number of uncharacterised DEGs (48%) might represent cnidarian or scleractinian-specific genes likely to be involved in the regulation of cnidarian/scleractinian-specific functions, such as symbiosis and calcification. According to Williams *et al*., (2009) conserved signaling pathways regulate the expression of taxon-specific genes during *Haliotis asinina* metamorphosis thus is likely that in corals conserved pathways not only regulate basic morphogenetic processes such apoptosis, protein synthesis, and cell migrations, they are also likely to control the expression of taxon-specific molecules responsible for taxon-specific variations. Despite this, it is possible as suggested by Grasso *et al*., (2008) that the number of taxon-restricted genes reflects the lack of genomic data for a vast range of non-model organisms and that some of these molecules have a wider distribution.

By contrast with the low annotation rate of transcripts differentially expressed in *M. faveolata* adults (16%)(Reyes-Bermudez *et al.,* in press), the percentage of annotated genes (from all annotated DEGs) in the adult *Acropora* cluster was 28%, which corresponded to 55% of all DEGs with in the cluster (CIV). This trend was observed in all clusters, while 52% of all sequences in CI were annotated, 44% were annotated in CII and 54% were annotated in CIII.

5. 5. 3. Genes down-regulated after settlement: Initiation of morphogenesis, calcium signalling and lipid metabolism related molecules.

Environmental cues are known to induce metamorphosis in a diverse range of marine invertebrate larvae (Hadfield *et al,* 2000); in most cases "inducers" are chemical compounds produced by marine microorganisms (Woollacott and Hadfield, 1996; Bonar and Fitt, 1990; Johnson and Giddins, 1991). It has been suggested that in corals, lectins may act as cell surface receptors to "inducers" (Grasso *et al.,* 2008) thus likely to represent the first step in the signalling cascade that will lead to metamorphosis in coral planula. Substrate recognition and initiation of metamorphosis in some anthozoans is achieved via a nerve-rich region located in the aboral pole of planulae known as the apical organ (Chia and Koss, 1979). This region, which contains a highly elaborate nerve network consisting of chemosensory neurons (Pang *et al.,* 2004) and three different types of sensory cells (Chia and Koss, 1979), is responsible for metamorphosis in the sea anemone *Nematostella vectensis* (Matus *et al.,* 2007; Rentzsch *et al.,* 2008) and the coral *Pocillopora damicornis* (Vandermeulen, 1974).

Furthermore, there is evidence of RFamide-positive neurons in the aboral end of *Acropora millepora* larvae (Hayward *et al.,* 2001), and LWamide, another neuropeptide known to mediate metamorphosis in cnidarians (Katsukura *et al.,* 2003 and 2004) was up-regulated in *M. faveolata* planulae (Reyes-Bermudez *et al.,* in press). It is known that planula migration and metamorphosis in the hydroid *Hydractina echinata* is regulated by an antagonistic mechanism involving the neuropetides LWamide and RFamide (Katsukura *et al.,* 2003 and 2004). According to this, is reasonable to think that metamorphosis in scleractinian corals is likely to be regulated by conserved mechanisms that resemble that described for hydrozoan cnidarians, and that neuropeptides such as RFamide and LWamide are involved in the translation of the signal, downstream the binding of metamorphosis "inducers" to specific aboral-ectodermal planulae extracellular receptors.

The role of neurotransmitters as signalling molecules of metamorphosis is consistent with the up-regulation in *Acropora* planula of three molecules likely to act as extracellular neuroactive ligand-receptors (PATH: ko04080)(Table 5.2). In cnidarians the integration of external stimuli to cellular activity is likely to be achieved via a complex network of neuronal interactions involving diverse neurotransmitters (Kass-

Simon and Pierobon, 2007) therefore, neurotransmitters and neuronal ligands up-regulated at the planula stage are candidate molecules to regulate and integrate the overall metamorphosis signal upon binding of specific extracellular "inducers". These observations suggest that aboral sensory neurons regulate settlement and/or metamorphosis in corals by releasing endogenous neurotransmitters as secondary massagers in response to binding of environmental cues to cell surface receptor proteins.

5. 5. 3. 1. Initiation of morphogenesis: BMP signalling.

The finding that 46% of all DEGs were down-regulated after settlement indicates that during metamorphosis and settlement gene expression changes rapidly due to intrinsic developmental signaling pathways likely to regulate both morphogenesis of adult structures and physiological responses to the new environment. The subset of genes that showed highest expression at the planula stage are likely to be involved in planula specific metabolic pathways such as lipid metabolisms (Table 5.2), as well as in the initiation of the morphogenetic changes experienced by *Acropora* larvae during the transition from a demersal, swimming, non-calcifying stage to a benthic, calcifying, sessile life style.

Metamorphosis is a morphogenetic process similar to gastrulation; in both cases tissues are reorganized and cell populations migrate and differentiate (Magie and Martindale, 2008). Grasso *et al.,* (2008) found that 52% of all DEGs were down regulated after *A. millepora* embryogenesis, which is consistent with the finding that a high percentage (46% of all DEGs) were down-regulated after settlement and the initiation of metamorphosis in this study. Among those DEGs we found an overrepresentation of positive regulators of apoptosis (GO:0043065) and larval development (GO:002164) as well as a number of components of conserved signalling pathways known to regulate apoptosis, cell differentiation, migration and proliferation across the Metazoa (Table 5.2). These results suggest that although the morphogenesis of adult structures is more evident after settlement, the regulation of metamorphic transitions might be modulated by transcripts generated at the planula stage and to some extend adult structures such as mesenteries are formed before

settlement (Vandermeulen, 1974; Babcock and Heyward, 1983; Szmant-Froelich *et al*., 1980).

Another interesting finding is the up-regulation in planulae of unigenes encoding *Acropora* orthologs of BMP-1 and BMP-1a molecules (Table 5.2). BMP-1 proteins belong to a small group of zinc and calcium dependent proteinases which activity is essential for tissue patterning and extracellular matrix assembly (Berry *et al*., 2009). In vertebrates, they are involved in the biosynthetic processing of extracellular matrix (ECM) precursors such as fibrillar collagens (Unsold *et al*., 2002; Medech *et al*., 2003) and proteoglycans (Scott *et al*., 2000; Ge *et al*., 2004) as well as in the regulation of the related family members BMP-2/4 (Jasuja *et al.,* 2007), which induce osteoblast differentiation (Wozney *et al.,* 1988) and their gradients are known to specified ventral and dorsal tissues (Dale and Wardle, 1999). By analogy with their vertebrate counterparts, it is reasonable to hypothesise roles for *Acropora* BMP-1 orthologs in processing ECM proteins and/or activating grow factors such as BMP-2/4 during coral metamorphosis. Cell-cell and cell-ECM interactions are fundamental processes regulating morphogenesis among metazoans (Magie and Martindale, 2008).

Components of the BMP signalling transduction pathway have been identified in *Acropora* (Samuel *et al.,* 2001; Hayward *et al.,* 2002) and the sea anemone *Nematostella* (Finnerty *et al.,* 2004; Rentzsch *et al.,* 2006). In both anthozoans, BMP signalling components are expressed asymmetrically in both the primary (oral-aboral axis as well as in a secondary axis perpendicular to this one known as the directive axis (Rentzsch *et al.,* 2006). Although these results have led to the suggestion of roles in dorso-ventral patterning in cnidarians (Hayward *et al.,* 2002; Rentzsch *et al.,* 2006), roles in cell differentiation and the establishment of morphogenetic borders also have been considered (Hayward *et al.,* 2002). The roles of BMP signalling in vertebrate skeletal formation are well characterized (Wozney *et al.,* 1988; Kobayashi *et al.,* 2005); ossification requires both activation of BMP-2/4 and processing of calcifying ECM proteins by BMP-1 (Ge and Greenspan, 2006). These observations suggest that as in the case of vertebrate skeletogenesis, BMP signaling might play an important role during calicoblast differentiation and coral skeleton deposition. The localization of a coral BMP-2/4 ortholog in the calcifying epithelium (Zoccola *et al.,* 2009) is

consistent with this idea. Up-regulation in planula (CI-EP7) of molecules involved in protein dimerization (GO:0042802) might reflect BMP activity because dimerization of matured BMP peptides is necessary to generate active ligands (Reber-muller *et al.,* 2006). Functional studies are necessary to test this idea.

There is evidence that in vertebrates wnt signaling regulates BMP-2 during chondrocyte differentiation (Gaur *et al.,* 2006) and that coordinate activation of notch, wnt and TGF-β signaling is necessary for BMP-2 mediated osteogenesis (Sciaudone *et al.,* 2003; Zamurovic *et al.,* 2004). The fact that components of some of these conserved signal transduction systems are up-regulated in planula larvae (Table 5.2) suggests that to some extent calicoblast differentiation pathways might resemble those described for the calcifying vertebrate cell lineages. This idea is consistent with the up-regulation of notch signaling components and regulators of BMP-2/4 activity during metamorphosis in the "robust" coral *M. faveolata* (Reyes-Bermudez *et al.,* in press). Functional assays need to be performed in corals to understand whether the roles and interactions between these conserved signaling systems resembled the ones described for vertebrates.

5. 5. 3. 2. Calcium signalling and lipid metabolism.

Eukaryotic organisms use changes in the concentration of intracellular calcium to regulate a diverse range of cellular processes (Haeseleer *et al.,* 2002; Carafoli, 2004). Differential cytosolic calcium concentrations are translated into cellular activities via calcium binding proteins known as calcium sensors. These molecules, upon calcium binding, are able to interact and activate target proteins, which activate specific calcium-dependent signaling cascades (Carafoli, 2003). Up-regulation in planulae of a number of EF-hand calcium sensors such as calmodulin, calumenin, calbindin, calreticulin and calcineurin (Table 5.2) suggest that calcium signalling is a key regulator during *Acropora* settlement and metamorphosis. These results are consistent with the up-regulation of calmodulin, calumenin and calreticulin orthologs in planula larvae of the coral *M. faveolata* (Reyes-Bermudez *et al.,* in press).

Up-regulation of calcium sensors in the planula larvae of two species that represents the two major scleractinian lineages (Kerr, 2005) suggest conserved calcium

dependent signalling pathways underlying the metamorphic transition across reef building corals. Calmodulin is a highly conserved calcium sensor known to regulate cytoskeleton organization, cellular metabolism, cell differentiation, proliferation, apoptosis and metamorphosis in eukaryotes (Cyert, 2001; Benaim and Villalobo, 2002; Vetter and Leclerc, 2003) therefore it is not surprising that this versatile calcium sensor modulates different aspects of settlement and metamorphosis across scleractinia. Up-regulation of calumelin and calreticulin in coral planula suggests that similar to mammalian bone homeostasis (Koga *et al.,* 2004), calcium signalling might regulate calicoblast differentiation and skeleton deposition in corals.

There is evidence that calumenin regulates the bone protein Osteocalcin and the transforming growth factor BMP-2 (Wajih *et al.,* 2004; Wajih *et al.,* 2006), which as mentioned before induces skeletogenesis across a wide range of organisms (Wozney *et al.,* 1988; Matsushiro and Miyashita, 2004) and localize to the calcifying epithelium in corals (Zoccola *et al.,* 2009). In the other hand, calreticulin belongs to the group of intracellular integrin ligands (Vallar *et al.,* 1999) and constituents of integrin-based adhesion complexes in vertebrates (Tran *et al.,* 2002), which suggest calcium dependent regulation of cell-ECM signal transduction mechanisms during coral metamorphosis. Cell migration, proliferation and differentiation are mediated by interactions between the actin-cytoskeleton (Hotchin and Hall, 1996) and surrounding ECM components (Magie and Martindale, 2008). This is consistent with the up-regulation in *Acropora* planulae of a number of transcripts involved in the regulation of the actin-cytoskeleton (PATH: ko04810)(Table 5.2) however; actin-cytoskeleton activity might also reflect active transport of calcifying ECM precursors or/and calcium ions prior skeleton deposition.

There is evidence that 1) calcium is accumulated in endodermal lipid containing vesicles in both planula and settled larvae of the aposymbiotic corals (Clode and Marshall, 2004) and 2) that the calcium binding fraction in the calcifying ECM of hermatypic corals is likely to be acidic phospholipids (Isa and Okasaki, 1987; Watanabe *et al.,* 2003). Acidic phospholipids are degradation products from lipid metabolism known to promote deposition of calcium salts (Khant *et al.* 1996). Roger and Thomas, (2001) suggested that ancestral endodermal lipid containing cells in cnidarians were used as physiological calcium sinks and that the coral skeleton

evolved from a waste removal mechanism subsequently adapted for structural support. According to this, lipid metabolism is not only likely to provide energy for planulae activity (Vandermeuden, 1974), acidic phospholipids produced as degradation products might represent a mechanisms by which calcium ions are insolubilized and transported to the calcificartion site prior skeleton deposition.

These ideas are consistent with the observed up-regulation in *Acropora* planulae of a number of DEGs involved in lipid metabolism and phospholipid biosynthetic process (GO:0008654)(Table 5.2). Microscopic observations identified putative lipid-containing vesicles in the endoderm of *Acropora* planula (data not shown), and Vandermeuden, (1974) described lipid-containing vesicles likely to be peroxisomes in the endoderm of planulae of the scleractinian coral *Pocillopora damicornis*. Peroxisomes are cellular organelles described for both animal and plant tissues, they are calcium-containing compartments involve in fatty acid metabolism, cholesterol synthesis, and phospholipid production (MacDonald *et al.,* 2004; Raychaudhury *et al.,* 2006). Furthermore, there is evidence demonstrating that peroxisomal stored calcium is used as a mediator towards calcium signalling (Raychaudhury *et al.,* 2006) and it is know that as result of lipid metabolism the secondary messengers diacyl-glycerol and IP_3 are produced (Hardie, 1991). These ideas are consistent with the up-regulation in planulae of components of the phosphatidylinositol signalling system (Table 5.2).

These results suggest that both lipid metabolism and calcium signalling are two main regulators of cellular activity in *Acropora* planulae. Calcium signalling might regulate cell differentiation, proliferation and migration as well as transport of calcium ions and calcifying ECM precursors during settlement and metamorphosis via a diverse range of calcium sensors. Lipid metabolism is likely to generate signalling molecules able to act as secondary messengers during settlement and metamorphosis as well as degradation products able to store and transport calcium prior skeleton deposition. Although lipid metabolisms seems to be the mayor energy source for planula activity, up-regulation in CI (Table 5.2) of a number of DEGs involved in carbohydrate metabolism (Table 5.2) suggests that lipids are not the only energy source in *Acropora* larvae.

5. 5. 4. Genes up-regulated in settled juvenile and calcifying stages: Morphogenesis of adult structures and skeleton deposition related molecules.

Among the DEGs up-regulated after settlement 84 (7% of all DEGs) showed the highest expression in calcifying stages (F, Po and T) (CII) and 243 (20% of all DEGs) in settled juvenile stages (CIII). While the DEGs up-regulated in juvenile settled polyps (CIII)(F and Po) reflected morphogenesis of adult structures the ones clustered in CII are consistent with roles in calcification. Coral skeleton deposition correlates with the aboral ectodermal transition from columnar cells to the calicoblast phenotype (Le Tessier, 1998; Clode and Marshall, 2004). This newly formed epithelium secretes a macromolecular network of proteins, lipids and polysaccharides known as the calcifying organic matrix (OM). Only calicoblastic cells in direct contact with the skeleton appear to be responsible for the synthesis and secretion of OM components (Puverel *et al.,* 2005).

5. 5. 4. 1. Calcification related molecules.

Calcifying OMs exert biological control over mineral deposition as they control $CaCO_3$ nucleation and determine the type of polymorph, as well as crystal size and shape (Fukuda *et al.,* 2003; Clode and Marshall, 2003; Watanabe *et al.,* 2003). Up-regulation in calcifying stages (CII) of a number of transcripts involved in O-Glycan biosynthesis (GO:0004553), metalloendopeptidase activity (GO: 0004222) and regulators of the tubulin-cytoskeleton (Table 5.2) suggest active OM synthesis. Matrix biosynthesis, rather than $CaCO_3$ deposition may be the limiting factor controling coral skeletogenesis (Allemand *et al.,* 1998). While glycosilation is a process that appears to be a prerequisite for skeleton formation in corals (Allemand *et al.* 1998) and echinoderms (Ameye *et al.,* 2001), cytoskeleton-dependent processes have been reported in calicoblastic cells and seem to be the mechanisms by which OM precursors are transported to the mineralization site in the coral *G. fascicularis* (Allemand *et al.,* 1998; Clode and Marshall 2002).

Furthermore, up-regulation of two types of carbonic anhydrases (CAs) and a carbonic anhydrase inhibitor in CII are consistent with the results reported by Grasso *et al.,* (2008) and the idea that DEGs cluster in this group are likely to have a role in skeleton deposition. Up-regulation of a CA inhibitor along side two CAs suggests that

CA activity is restricted to distinct cell population such as the calicoblastic epithelium (Grasso *et al.,* 2008). CAs catalyse the reversible conversion of CO_2 to HCO^-_3, which is one of the substrates necessary for $CaCO_3$ deposition (Tambutté *et al.* 1996; Allemand *et al.* 2004). CA activity has been link to calcification in the azooxanthellate coral *Tubastrea aurea* (Tambutté *et al.,* 2007), the calicoblastic ectoderm of the zooxanthellate coral *Stylophora pistillata* (Moya *et al.,* 2008) and in the calicoblastic ectoderm of *Acropora hebes* (Isa and Yamazato, 1984). Furthermore, a role for CA in skeletogenesis has been demonstrated in the coralline demosponge *Astrosclera willeyana* (Jackson *et al.* 2007).

Up-regulation in the calcification cluster (CII), of components of the TGF-β and MAPK signal transduction pathways (Table 5.2) suggest that these conserved signalling systems are likely to regulate calicoblast differentiation or/and transport/synthesis of OM precursors during coral skeleton deposition. Likewise, uncharacterised molecules in this cluster (CII) are candidate molecules for roles in calcification as structural OM components or regulators of OM synthesis.

5. 5. 4. 2. Morphogenesis of adult structure related molecules.

Although the subset of DEGs clustered in CIII might reflect the metamorphic transition that will lead to the formation of adult polyps, expression patterns (EPs) within the cluster revealed that F and Po have distinct transcription profiles. While CIII-EP3, 4 and 7 contain candidate genes to regulate morphogenesis and juvenile skeleton deposition as shown by the over representation of the GO categories: transcription factor activity (GO:0003700), embryonic development ending in birth or egg hatching (GO:0009792), regulation of anatomical structure morphogenesis (GO:0022603), cation transport (GO:0006812) and anion transport (GO:0006820); CIII-EP1 contained 52 DEGs that were only up-regulated in Po and CIII-EP-2 grouped 75 DEGs that were only up-regulated in F (Fig 5.3).

These results suggests that even though F and Po co-expressed a subset of genes likely to regulate the morphogenetic processes that will lead to the formation of adult structures DEGs within CIII-EP1-2 might be involved in stage specific processes. Up-regulation in F of transcripts encoding the Galaxin-like1 protein (Table 5.2) which is

predicted to be extracellular and whose expression pattern is consistent with a role in basal plate formation (chapter 3), together with up-regulation in Po of sequences matching the ECM proteins echinonectin and E-selectin suggests overlapping expression of extracellular matrix proteins between the two juvenile settled stages used in this study. Whether the role of these ECM proteins is to differentially regulate skeleton deposition and/or cell adhesion during morphogenesis of adult structures is not clear. Functional assays are necessary to understand the function of these secreted proteins as well as the specific role of uncharacterised transcripts up-regulated in settled juvenile stages during *Acropora* metamorphosis.

Upregulation in F (CIII-EP2) of positive regulators of cell differentiation (GO:0045597)(Table 5.2) suggests that cell differentiation into adult phenotypes is regulated to some extent by transcripts originated in F. On the other hand, up-regulation in Po (CIII-EP1) of two DEGs (cyclin b3 and polymerase delta), orthologs of which have roles in cell proliferation (PATH: ko041100 and PATH: ko03030) suggests that cell division is actively occurring in Po. According to this, is reasonable to think that whilst morphogenesis of adult structures (i.e. mesenteries, tentacles and the calicoblastic ectoderm) is mediated by transcripts originated in F, cell proliferation of committed cell populations occurs in Po prior asexual reproduction and/or growth.

In a similar way, up-regulation in F (CIII-EP2) of DEGs involved in cellular carbohydrate metabolic processes (GO:0044262) and aerobic respiration (GO:0009060) indicates a switch between energy metabolic pathways. While the main energy source for planula larva is stored lipid (Vandermeulen, 1974), these results are consistent with carbohydrate metabolism being the main energy source of settled juvenile polyps. The switch between energy metabolic pathways between planulae and settled juvenile polyps might also reflect a change in the concentration of the secondary messengers regulating different aspects of *Acropora* metamorphosis. While lipid metabolism is able to generate the secondary messengers such as diacyl-glycerol and IP_3 (Hardie, 1991), an increase in respiration (GO:0009060) rates due to a boost in carbohydrate metabolism (GO:0044262) will raise the concentration of reactive oxigen species (ROS), which are molecules able to regulate specific signalling pathways (Finkel, 2000; Brookes et al. 2002; Dennery, 2007). Up-

regulation of molecules involved in oxidoreductase activity (GO:0016491), response to oxidative stress (GO:0006979) in both F and Po that are also down-regulated in adult polyps (CIII-EP3 and 7) are consistent with this idea.

This idea is consistent with the results reported for the coral *M. faveolata* (Reyes-Bermudez *et al.,*in press). Over representation of GO categories: oxidoreductase activity (GO:0016491) and response to oxidative stress (GO:0006979) in CIII-EP3-7 suggests that ROS produced by aerobic respiration (GO:0009060) are involved in the regulation of *Acropora* metamorphosis in settled juvenile stages. Incomplete oxygen reduction during the mitochondrial electron transport chain leads to the formation of superoxide radicals which are the first molecules involved in the production of ROS (Kang and Hamasaki, 2003; Raha and Robinson, 2000). Uncontrolled ROS production can cause oxidative damage to cellular structures (Raha and Robinson, 2000), however ROS molecules are also able to regulate specific signalling pathways (Finkel, 2000; Brookes et al. 2002). ROS plays a direct role as a primary or secondary messenger during embryonic development in mammals by regulating cellular pathways involved in cell proliferation, differentiation and apoptosis (reviewed in Dennery, 2007).

Another result that supports this idea is the down-regulation of a subset of GFP-like encoding transcripts following *Acropora* settlement (GO:0008218)(Table 5.2). Coral GFPs have been associated to oxidative stress response due to their ability to quench ROS (Bou-Abdallah *et al.,* 2006; Smith-Keune and Dove, 2008), which suggests that up-regulation of GFPs in planulae might represent one mechanism by which ROS levels are regulated. Although there is no direct evidence that down-regulation of a subset of GFPs after settlement leads to oxidative stress in the settled juvenile *Acropora,* the fact that the GO categories oxidoreductase activity (GO:0016491) and response to oxidative stress (GO:0006979) are up-regulated in juvenile settled stages following down-regulation of GFPs (GO:0008218) supports this idea. Moreover, up-regulation of a subset of GFPs in Pla relative to all stages (CI-EP1) suggests a specific function for these molecules prior settlement and metamorphosis in *Acropora.* Functional assays are necessary to understand the role of larval GFPs and their putative role as ROS regulators.

In contrast with results reported for the "Robust" coral *M. faveolata* (Reyes-Bermudez *et al.,* submitted), over representation of molecules involved in responses to oxidative stress (GO:0006979) were observed in the "Complex" coral *A. millepora* in settled juvenile stages rather than in planula larvae. These observations suggest variation in the cellular mechanisms regulating metamorphic morphogenesis across reef-building corals that might reflect phylogenetic history. The order Scleractinia is composed of two main clades (i.e. Robust/Short and Complex/Long) that are thought to have diverged approximately 240-288 years ago (Kerr, 2005; Medina et al. 2006, Romano and Palumbi, 1997). Marlow and Martindale (2007) reported different gastrulation mechanisms between scleractinian clades, thus is not surprising that variation in the mechanisms regulating settlement and metamorphosis exist within the order. More research is necessary to validate these observations.

5. 5. 5. Cluster IV. Genes with highest expression in adult tips: asexual reproduction and genes associated with responses to environmental conditions.

Consistent with the idea that *Acropora* adult tips have a higher growth rate that other parts of the colony (Oliver et al., 1983; Tunnicliffe et al., 1983), our results show an over representation in CIV-EP1 of DEGs involved in translation (GO:0006412), pattern specification process (GO:0007389) and regulation of cell differentiation (GO:0045595) (Fig 5.5). Furthermore, up-regulation in the same EP of the coral orthologs of snail, beta-catenin and notch-like (Table 5.2), which are known regulators of developmental processes (Technau and Scholz, 2003; Cadigan and Nusse, 1997; Gordon, 2008), implies that cellular differentiation is also occurring. Up-regulation in CIV-EP1 of regulators of the actin cytoskeleton (PATH: ko04810) such as profilin, actin and gelsolin (Table 5.2) is likely to reflect interactions between the actin-cytoskeleton and surrounding ECM components during cell migration, proliferation and differentiation (Hotchin and Hall, 1996; Magie and Martindale, 2008).

Over-representation of the GO category: anatomical structure development (GO:0048856) in all post-settlement stages (CIV-EP4) suggests that this EP represents the gene network underlying polyp morphogenesis during development, asexual reproduction and growth. These results imply that similar regulatory gene

networks regulate the formation of adult structures during metamorphosis and asexual reproduction in *Acropora*. Consistent with this, many genes are expressed in two waves during *Drosophila melanogaster* development, larval patterns being recapitulated in adults, where they had analogous regulatory roles (Arbeitman *et al.,* 2002). By analogy, the same genetic programming may be used in *Acropora* to generate a polyp during metamorphosis and during asexual reproduction in the adult colony. This idea is consistent with the results for *M. faveolata* metamorphosis where the planula transcriptome resembled that of the adult (Reyes-Bermudez *et al.,* in press).

The fact that the developmental regulatory DEGs clustered in CIV-EP1 were not differentially expressed in settled juvenile stages relative to planulae but were up-regulated in adult tips might be explained by: 1) high metabolic rates characteristic of symbiotic coral tissues (Stanley, 2006) or/and 2) modifications between the developmental pathways that originates polyps during development and asexual reproduction. Although more research is necessary to understand co-expressed gene networks in contrasting life stages, these results suggest that to some extent similar cellular mechanisms might control polyp morphogenesis during metamorphosis and asexual reproduction.

Consistent with the idea that co-expressed gene networks are used in different life stages during the life history of an organisms (Arbeitman *et al.,* 2002), expression patterns CIV-EP2, 3 and 5 clustered DEGs that had their highest expression in adults but were also up-regulated in F, Po and Pla respectively (Fig 5.5). While the group of DEGs that were up-regulated in both F and T (CIV-EP2) contains DEGs involved in responses to abiotic stimuli (GO:0009628), such as heat shock proteins and cryptochromes; the genes up-regulated in Po and T were involved in skeletal-system development (GO:0001501), extracellular matrix production (GO:0044420) and defence responses (GO:0006952) (Table 5.2). These results suggest that whereas DEGs up-regulated in both F and T might reflect the physiological responses to the sessile life style, the DEGs up-regulated in both Po and T reflect shared calcification mechanisms between primary and adult polyps as well as immune responses likely to be involved in the establishment of symbiosis.

The finding that calcification-related transcripts were up-regulated both in Po and T (CIV-EP3) is consistent with the idea that the fiber-like $CaCO_3$ deposition characteristic of adult skeletogenesis (Vandermuelen and Watabe, 1973; Goreau and Hayes, 1977; Gladfelter, 1982) begins once mesenteries are formed in the polyp stage (chapter 3). Moreover, these results are consistent with the ideas that 1) juvenile calcification in *Acropora* is a two-stage process that involves the formation of a basal plate in F followed by septa deposition and complete corallite formation in Po and 2) that this two stages are regulated by differential expression of OM components (chapter 3).

In a similar way, the finding that a CaM-like molecule which appears to be coral specific and interacts *in vitro* with the *Acropora* CaM protein (chapter 4) clustered together with alpha type 1 collagen and fibroblast growth factor receptor coral orthologs (CIV-EP3) (Table 5.2), suggests that CaM mediated calcium signalling regulates calicoblast differentiation in *Acropora* and that the translation of the calcium signal is mediated via a coral specific calcium sensor. Alpha type 1 collagen is one of the major structural components of vertebrate bone (Ge and Greenspan, 2006) and fibroblast growth factor receptor signaling mediates osteogenic differentiation in mammals (Selfors *et al.,* 1998; Ng *et al.,* 2007). These observation are consistent with the idea that diversification of calcium sensors had a crucial role in the evolution of taxon-specific calcium mediated signaling pathways (chapter 4).

On the other hand, up-regulation in both Pla and T (CIV-EP5) of DEGs encoding ADAM metalloproteinase and frizzled-related proteins (Table 5.2) whose vertebrate counterparts are involved in processing ECM proteins (Greenspan, 2005; Greenspan and Wang 2005) and Wnt signaling respectively (Cadigan and Nusse, 1997), suggest that cell differentiation into precursors of adult cell types is occurring in both planulae and adult tissues. While ECM components are able to mediated cell migration, proliferation and differentiation (Hotchin and Hall, 1996; Magie and Martindale, 2008), Wnt signalling plays an important role in the specification of cells fates during animal development (Cadigan and Nusse, 1997). Furthermore, cnidarians posses at least eleven from the twelve Wnt subfamilies known to exist in bilaterians and the expression patterns for these molecules revealed distinct roles during development in the sea anemone *Nematostella vectensis* (Kusserow *et al.,* 2005).

According to this is reasonable to think that DEGs grouped in CIV-EP5 might be involved to some extent in the establishment of cellular fates during both metamorphosis and asexual reproduction. Furthermore it is possible that cell differentiation into adult phenotypes is achieved in two steps: 1) generation of partially committed precursors of adult cell types as in the case of hydrozoans (Martin and Archer, 1997; Bode, 1996) and 2) final differentiation into specific cell types (chapter 2). The finding that Wnt signaling components are up-regulated both in Pla and T, together with the fact that frizzled-related proteins are Wnt receptors known to regulate canonical Wnt signalling during BMP-2 induced chondrocyte differentiation in mammals (Gaur *et al.,* 2006) suggests that calicoblast precursors are likely originated in *Acropora* planulae prior to metamorphosis and that final differentiation into the adult calcifying phenotype is achieved in Po after metamorphosis and during asexual reproduction in T.

This idea is consistent with the over representation in both Po and T (CIV-EP3) of DEGs involved skeletal-system development (GO:0001501) and extracellular matrix production (GO:0044420) as well as with the up-regulation in T (CIV-EP1) of an *Acropora* beta-catenin ortholog which is the downstream protein that regulates gene expression of target genes during canonical Wnt signalling (Cadigan and Nusse, 1997). Although gene expression microarray analysis is a valuable tool to identify key molecules and specific gene networks regulating many aspects of coral biology, the functions and physiological interactions of the genes identified by this approach are restricted to conceptual models based on analogous pathways in model organisms. Functional assays are necessary to validate the models and hypothesis generated from microarrays experiments in scleractinian corals.

5. 6. CHAPTER FIGURES AND TABLES.

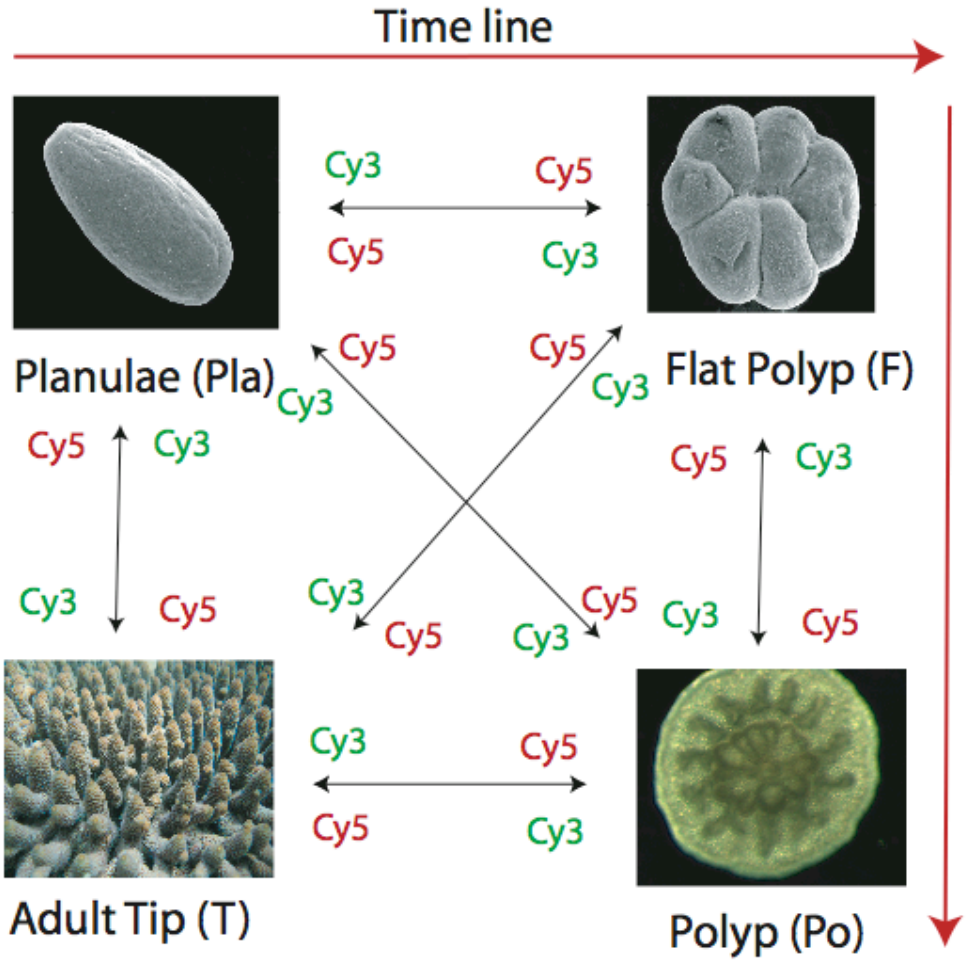

Figure 5. 1. Experimental design.

Gene expression microarray analysis was used to compare changes in *Acropora millepora* transcriptome between aposymbiotic non-calcifying planula (Pla) larvae, aposymbiotic calcifying flat polyp (F), aposymbiotic calcifying primary polyp (Po) and symbiotic calcifying adult tips (T). Samples were hybridized in duplicate fallowing a loop design. Hybridizations included dye swaps between all stages in both biological replicates.

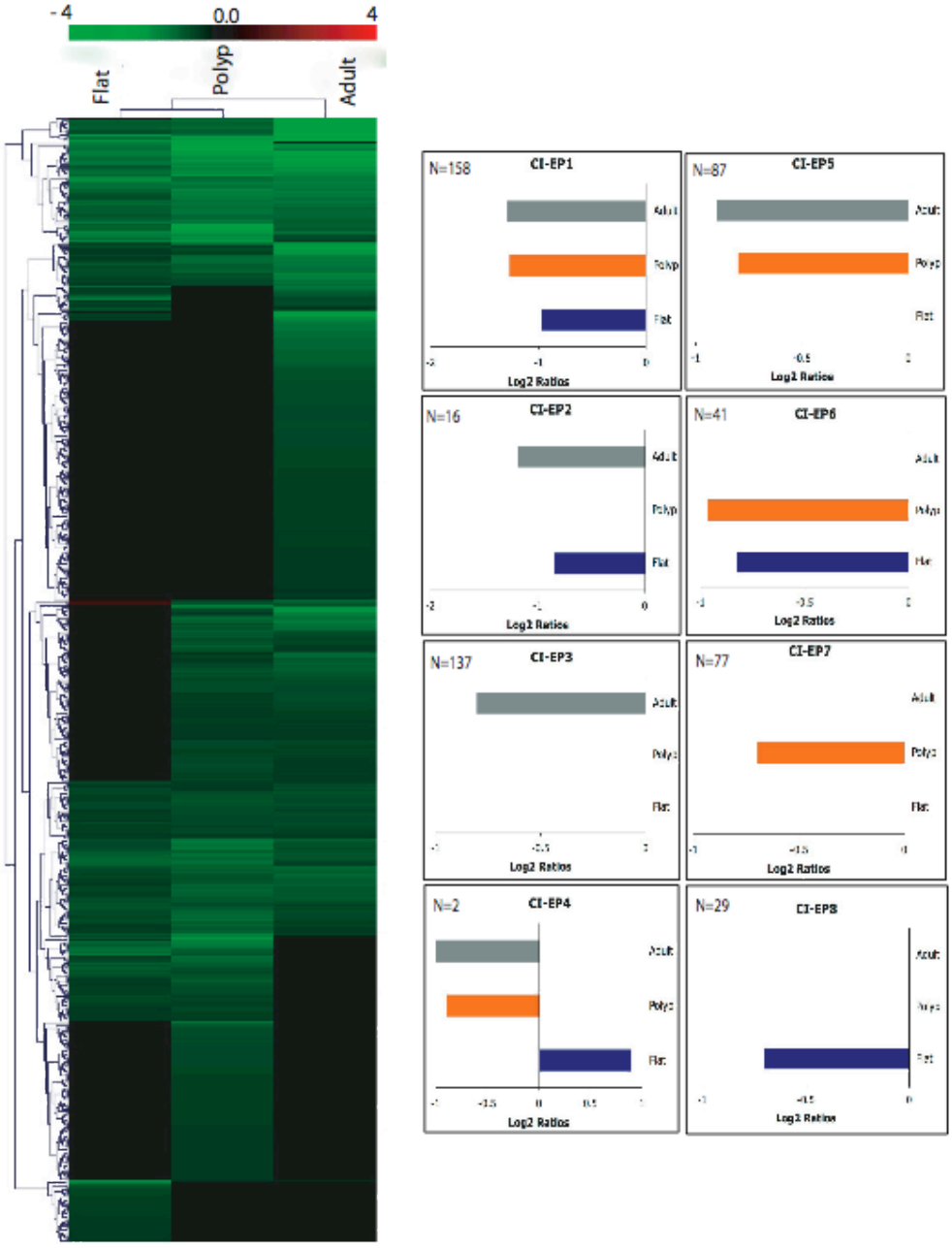

Figure 5. 2. Cluster I: DEGs downregulated after settlement.

Cluster I consisted of 547 unigenes (46% of DEGs) that showed the highest expression at the planula stage. Expression values are presented as the log2 intensity ratio (M-value) between all post-settlement stages and planulae (baseline). Absolute fold changes (FCs) in CI were as high as ~10-fold (~3.3 log2 ratio). Eight distinct expression patterns were identified within the group (CI-EP1 to CI-EP8). Expression patterns show the stage average log2 ratio for all DEGs with similar expression profiles.

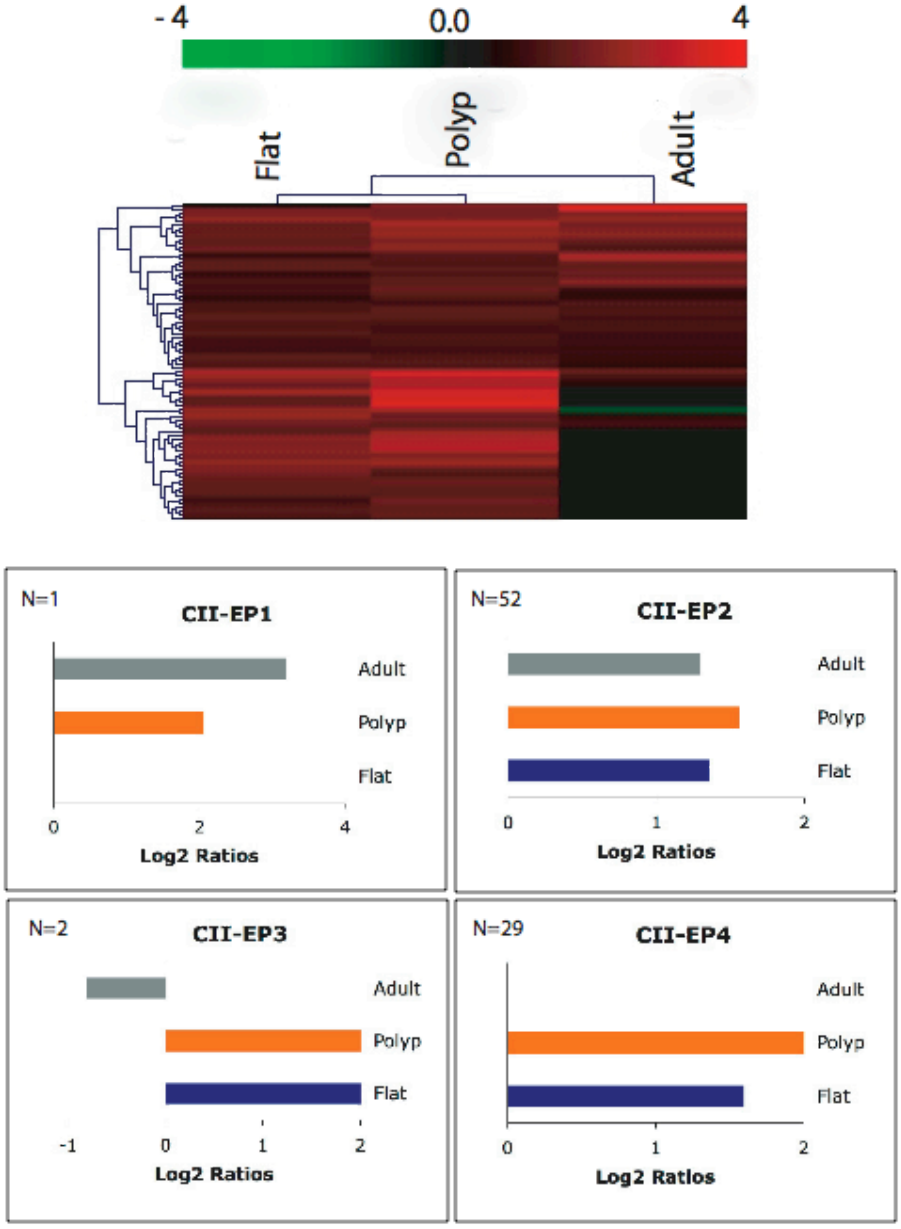

Figure 5. 3. Cluster II: DEGs upregulated after settlement.

Cluster II consisted of 84 unigenes (7% of DEGs) that showed the highest expression in calcifying stages. Expression values are presented as the log2 intensity ratio (M-value) between all post-settlement stages and planulae (baseline). Absolute fold changes (FCs) in CIII were as high as ~14-fold (~3.8 log2 ratio). Four distinct expression patterns were identified within the group (CII-EP1 to CII-EP4). Expression patterns show the stage average log2 ratio for all DEGs with similar expression profiles.

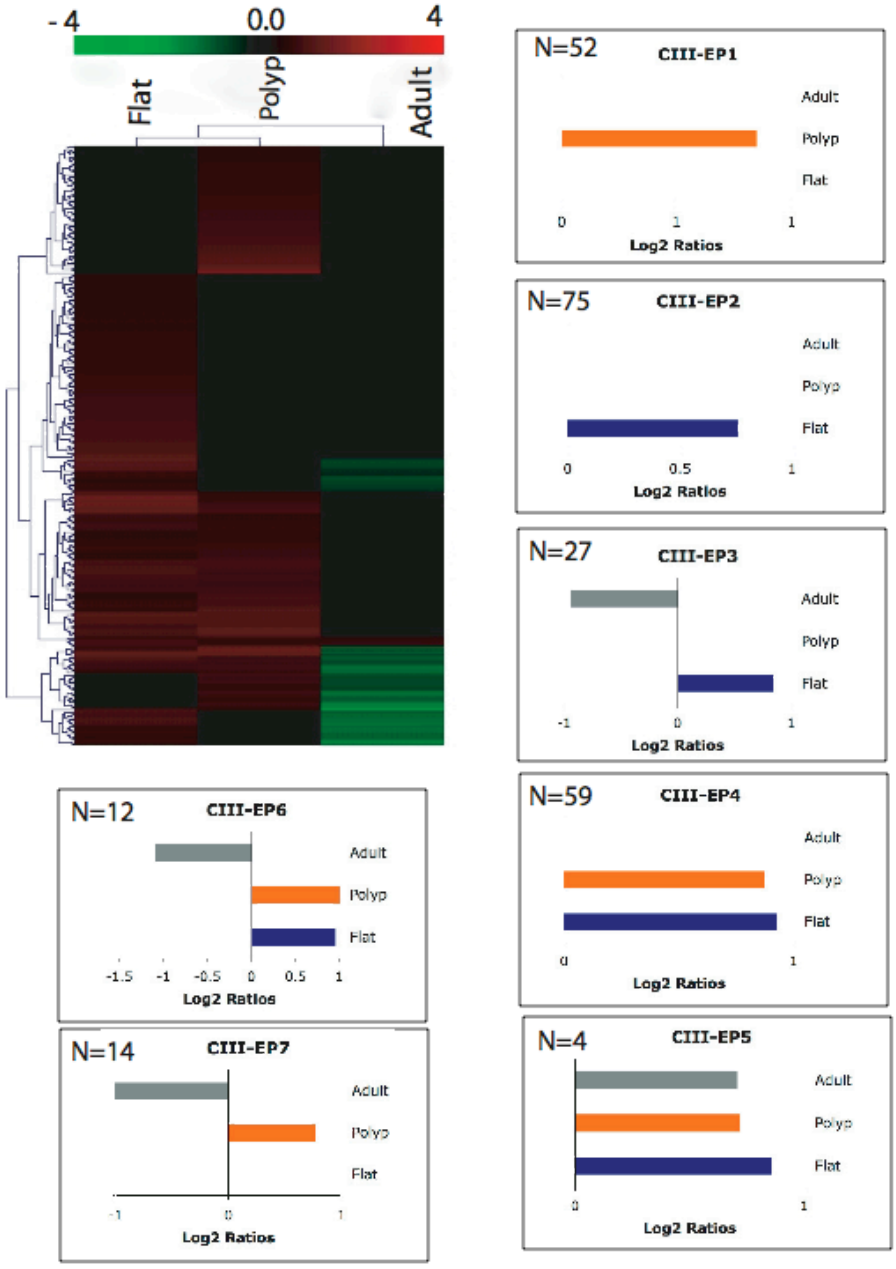

Figure 5. 4. Cluster III: DEGs with highest expression in juvenile settled stages.

Cluster III consisted of 243 unigenes (20% of DEGs) that showed the highest expression in juvenile settled stages. Expression values are presented as the log2 intensity ratio (M-value) between all post-settlement stages and planulae (baseline). Absolute fold changes (FCs) in CIII were as high as ~4-fold (~2 log2 ratio). Seven distinct expression patterns were identified within the group (CIII-EP1 to CIII-EP7). Expression patterns show the stage average log2 ratio for all DEGs with similar expression profiles.

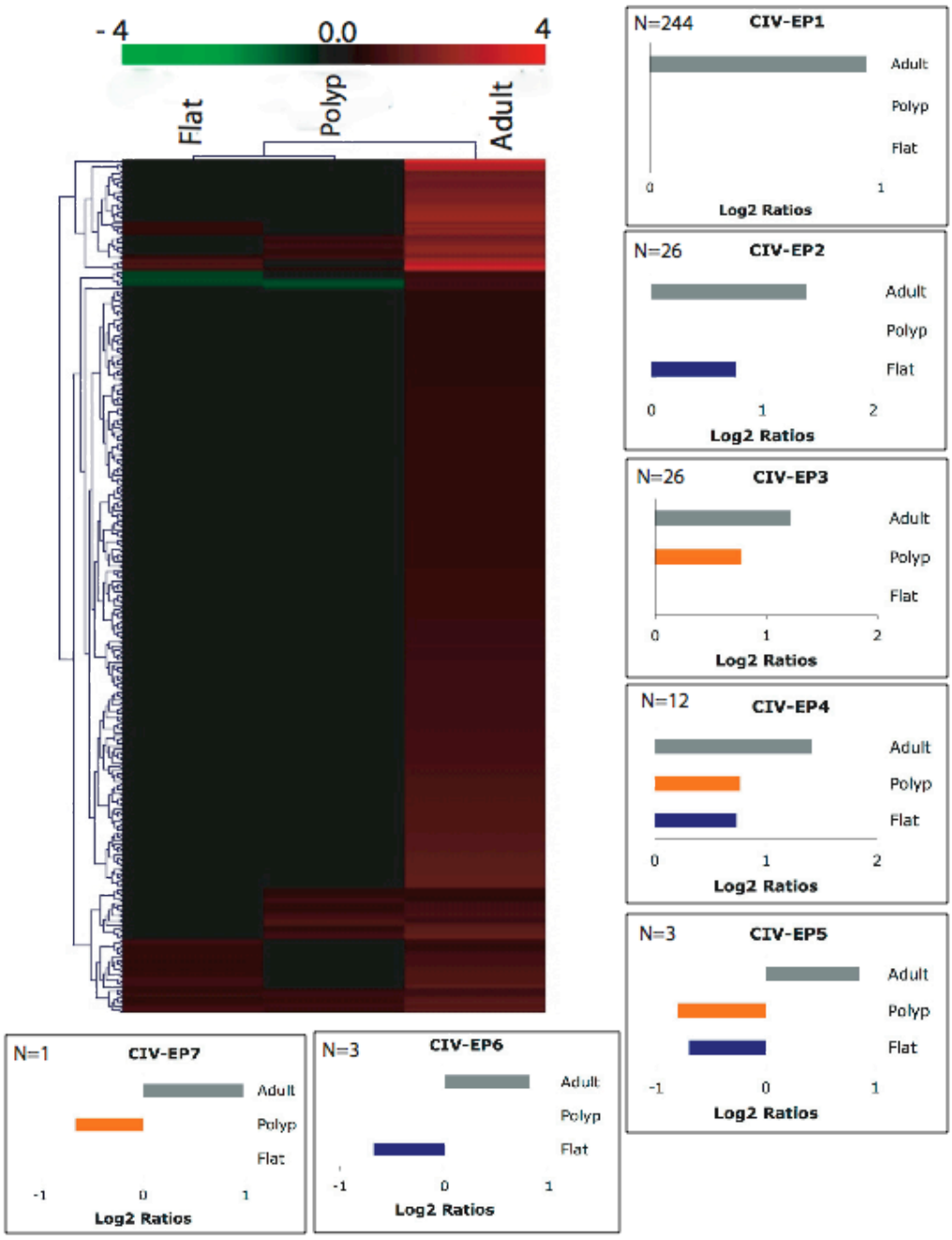

Figure 5. 5. Cluster IV: DEGs with highest expression in Adult tips.

Cluster IV consisted of 315 unigenes (27% of DEGs) that showed the highest expression in adult tips. Expression values are presented as the log2 intensity ratio (M-value) between all post-settlement stages and planulae (baseline). Absolute fold changes (FCs) in CIV were as high as ~20-fold (~4.3 log2 ratio). Seven distinct expression patterns were identified within the group (CIV-EP1 to CIV-EP7). Expression patterns show the stage average log2 ratio for all DEGs with similar expression profiles.

Table 5. 1. Cluster and expression patterns summary.

Cluster	Assignment	Expression Pattern	# of DEGs	% of DEGs	% of Annotated DEGs (623 genes-52% of DEGs)
CI	Downregulated after settlement.	CI-EP1	158	13.3 %	10.4 % (65 genes)
		CI-EP2	16	1.3 %	0.1 % (6 genes)
		CI-EP3	137	11.5 %	12.5 % (78 genes)
		CI-EP4	2	0.2 %	0.3 % (2 genes)
		CI-EP5	87	7.3 %	6.1 % (38 genes)
		CI-EP6	41	3.4 %	3.2 % (20 genes)
		CI-EP7	77	6.5 %	9.5 % (59 genes)
		CI-EP8	29	2.4 %	2.6% (16 genes)
CI Total			**547**	**46 %**	**45 % (284 genes)**
CII	Upregulated after settlement.	CII-EP1	1	0.08 %	0.2 % (1 gene)
		CII-EP2	52	4.4 %	4 .6 % (28 genes)
		CII-EP3	2	0.1 %	0 %
		CII-EP4	29	2.4 %	1.2 % (8 genes)
CII Total			**84**	**7 %**	**6 % (37 genes)**
CIII	Upregulated in either/both Flat and Polyp and downregulated /stable in Adult	CIII-EP1	52	4.3 %	3.6% (23 genes)
		CIII-EP2	75	6.3 %	7 % (44 genes)
		CIII-EP3	27	2 %	3 % (19 genes)
		CIII-EP4	59	4.9 %	5 % (31 genes)
		CIII-EP5	4	0.3 %	0.2 % (1 gene)
		CIII-EP6	12	1 %	0.8 % (5 genes)
		CIII-EP7	14	1.2 %	1.4 % (9 genes)
CIII Total			**243**	**20 %**	**21 % (132 genes)**
CIV	Upregulated in Adult	CIV-EP1	244	20.5 %	21 % (133 genes)
		CIV-EP2	26	2.2 %	2.7 % (17 genes)
		CIV-EP3	26	2.2 %	2.3 % (14 genes)
		CIV-EP4	12	1 %	0.8 % (5 genes)
		CIV-EP5	3	0.3 %	0.3 % (2 genes)
		CIV-EP6	3	0.3 %	0.5 % (3 genes)
		CIV-EP7	1	0.08 %	0 %
CIV Total			**315**	**27 %**	**28 % (172 genes)**
Overall Total			**1189**	**100 %**	**100 %**

Table 5. 2. Functional annotation and expression values for DEGs of interest.

Unigene ID	Annotation	C-EP	GO/KEGG Term	FC-F	FC-Po	FC-T
CI						
C_D050-F6_46	ef-hand domain containing 2	CI-EP1	calcium ion binding	-1.65	-2.33	-1.72
C_GS01dC08	synaptic 2	CI-EP1	Biosynthesis of unsaturated fatty acids	-3.41	-2.30	-2.80
C_mge-B045-G10-prawn78	glucose-methanol-choline oxidoreductase	CI-EP1	Bile acid biosynthesis	-2.26	-2.32	-1.86
C_mge-C006-A7-pre48_T3	Colorless GFP-like protein	CI-EP1	---NA---	-2.33	-5.16	-2.18
C_mge-C007-D6-pre43_T3	pancreatic lipase-related protein 2	CI-EP1	Glycerolipid metabolism	-2.48	-3.94	-4.57
S_GS01SB11	Notch 2 (Fragment)	CI-EP1	Notch signaling pathway	-1.61	-1.67	-1.76
S_MGE-A049-C6-post42-T3	bone morphogenetic protein	CI-EP1	---NA---	-1.50	-2.27	-2.16
S_mge-B018-G8-prawn62_T3	family member k	CI-EP1	Glycerolipid metabolism	-1.74	-1.88	-1.89
S_mge-C005-A7-pre48_T3	Lipase, gastric	CI-EP1	Glycerolipid metabolism	-1.91	-1.51	-4.11
S_mge-C008-F10-pre77_T3	hemicentin 1	CI-EP1	Bioluminescence	-2.11	-2.64	-2.30
S_mge-C008-H4-pre31_T3	paired-like homeodomain TF-2	CI-EP1	TGF-beta signaling pathway	-2.22	-2.66	-2.60
S_mge-C012-B5-pre33_T3	red fluorescent protein2	CI-EP1	Bioluminescence	-2.43	-6.87	-2.68
S_MGE-C018-B4-pre25_T3	Orphan G protein-coupled receptor Ren 1	CI-EP1	Neuro ligand-receptor interaction	-2.06	-3.18	-3.46
S_MGE-C018-E7-pre52_T3	gdp-mannose-dehydratase	CI-EP1	Fructose and mannose metabolism	-1.55	-2.00	-1.56
C_GS01IG06	animal haem peroxidase	CI-EP2	alpha-Linolenic acid metabolism	-1.63	1.00	-1.77
S_mge-C015-H12-pre95_T3	sphingomyelin phosphodiesterase	CI-EP2	Sphingolipid metabolism	-1.56	1.00	-3.56
C_D012-C11_83	delta 5 fatty acid desaturase	CI-EP3	Linoleic acid metabolism	1.00	1.00	-1.64
C_MGE-A022-E1-post4-T3	calreticulin	CI-EP3	calcium ion binding	1.00	1.00	-1.48
S_GS01cE06	phosphatidylinositol-3-phosphate 5-kinase	CI-EP3	Phosphatidylinositol signaling system	1.00	1.00	-1.53
S_MGE-A006-D4-28927-T3	acyl-oxidase	CI-EP3	Fatty acid metabolism	1.00	1.00	-1.53
S_mge-A030-A4-post24-T3	ribokinase	CI-EP3	Pentose phosphate pathway	1.00	1.00	-1.52
S_mge-B019-C1-prawn2_T3	rx3 protein	CI-EP3	TGF-beta signaling pathway	1.00	1.00	-1.52
S_mge-B021-G8-prawn62_T3	Cytochrome P450	CI-EP3	Linoleic acid metabolism	1.00	1.00	-1.86
S_mge-C001-E10-pre76_T3	egf-like protein	CI-EP3	Notch signaling pathway	1.00	1.00	-2.60
C_GS01QA12	endonuclease	CI-EP5	Apoptosis	1.00	-1.67	-2.91
C_mge-A041-C3-post18-T	pancreatic lipase	CI-EP5	Glycerolipid metabolism	1.00	-1.67	-3.67
C_mge-B022-H10-prawn79	Bone morphogenetic protein 1a	CI-EP5	---NA---	1.00	-1.56	-1.71
C_mge-C006-A6-pre40_T3	calumenin	CI-EP5	calcium ion binding	1.00	-2.19	-8.11
C_mge-C007-E3-pre20_T3	gamma-aminobutyric acidaalpha 6	CI-EP5	Neuro ligand-receptor interaction	1.00	-1.72	-1.63

S_mge-A041-B5-post33-T3	triosephosphate isomerase	CI-EP5	Glycolysis / Gluconeogenesis	1.00	-1.94	-1.82
S_mge-A041-F10-post77-T3	25-hydroxyvitamin D-1-alpha-hydroxylase	CI-EP5	Biosynthesis of steroids	1.00	-1.55	-3.10
s_mge-b025-b6-prawn41_t3	bone morphogenetic protein 1	CI-EP5	---NA---	1.00	-1.55	-1.66
S_mge-C010-B6-pre41_T3	Thrombospondin 1	CI-EP5	TGF-beta signaling pathway	1.00	-1.50	-1.62
S_mge-C015-G2-pre14_T3	Aristaless	CI-EP5	---NA---	1.00	-1.82	-1.80
C_D018-B3_18	protein disulfide isomerase	CI-EP6	larval development	-1.66	-1.64	1.00
C_D049-F8_62	lipocalin protein	CI-EP6	larval development	-1.51	-1.54	1.00
C_mge-A043-C5-post34-T	phosphatidylserine decarboxylase	CI-EP6	phospholipid biosynthetic process	-2.35	-2.05	1.00
C_mge-C011-F7-pre53_T3	beta 10	CI-EP6	Regulation of actin cytoskeleton	-2.11	-1.82	1.00
C_mge-C014-C3-pre18_T3	calbindin 2	CI-EP6	calcium ion binding	-2.51	-2.11	1.00
S_mge-C001-H8-pre63_T3	phosphatidylserine decarboxylase-related	CI-EP6	phospholipid biosynthetic process	-2.83	-1.89	1.00
S_MGE-C017-G9-pre70_T3	pyruvate dehydrogenasealpha 1	CI-EP6	Glycolysis / Gluconeogenesis	-1.53	-1.61	1.00
C_D022-B6_42	mannose-isoform cra_a	CI-EP7	identical protein binding	1.00	-1.53	1.00
C_GS01KH06	arp2 3 complex 20 kd subunit	CI-EP7	Regulation of actin cytoskeleton	1.00	-1.57	1.00
C_MGE-A035-E1-post4-T3	gdp-mannose-dehydratase	CI-EP7	Fructose and mannose metabolism	1.00	-1.53	1.00
C_mge-A044-E12-post92-	cardiac calumenin	CI-EP7	calcium ion binding	1.00	-1.61	1.00
C_mge-B019-B3-prawn17_	dentin matrix protein 1	CI-EP7	---NA---	1.00	-1.57	1.00
S_GS01aE06	dipeptidyl-peptidase 7	CI-EP7	identical protein binding	1.00	-1.51	1.00
S_GS01HD01	heart and neural crest derivatives 2	CI-EP7	identical protein binding	1.00	-1.54	1.00
S_mge-A037-F5-post37-T3	casp8 and fadd-like apoptosis regulator	CI-EP7	Apoptosis	1.00	-1.68	1.00
S_mge-A038-H1-post7-T3	mitogen-activated protein kinase 7	CI-EP7	MAPK signaling pathway	1.00	-1.79	1.00
S_mge-B016-E5-prawn36_T3	8-lipoxygenase-allene oxide synthase fusion	CI-EP7	Arachidonic acid metabolism	1.00	-1.73	1.00
S_mge-C007-D7-pre51_T3	gamma glutamyl transpeptidases	CI-EP7	Arachidonic acid metabolism	1.00	-1.60	1.00
S_mge-C007-F7-pre53_T3	Allatostatin G-protein coupled receptor	CI-EP7	Neuro. ligand-receptor interaction	1.00	-1.58	1.00
S_mge-C009-A8-pre56_T3	alanine:glyoxylate aminotransferase	CI-EP7	identical protein binding	1.00	-1.51	1.00
S_mge-C009-H2-pre15_T3	calcineurin subunit b	CI-EP7	MAPK signaling pathway	1.00	-1.55	1.00
C_D003-D2	cytoplasmic actin	CI-EP8	Regulation of actin cytoskeleton	-1.59	1.00	1.00
C_GS01JG02	calmodulin1	CI-EP8	regulation of transport	-1.86	1.00	1.00
C_GS01WD05	myl9 protein	CI-EP8	Regulation of actin cytoskeleton	-1.55	1.00	1.00
C_mge-B023-F9-prawn69_	protein kinasebeta	CI-EP8	regulation of transport	-1.63	1.00	1.00
S_mge-A041-G5-post38-T3	phosphatidylinositol 4-alpha polypeptide	CI-EP8	Phosphatidylinositol signaling system	-1.55	1.00	1.00
CII						
S_MGE-A023-D8-post59-T3	major yolk protein	CII-EP1	iron homeostasis	1.00	4.14	9.06

C_D008-B12	hemolytic lectin cel-iii	CII-EP2	O-Glycan biosynthesis	2.94	4.99	4.46
C_MGE-A009-D7-57751-T3	ricin b lectin	CII-EP2	O-Glycan biosynthesis	2.83	4.51	3.78
C_MGE-A049-E7-post52-T	secreted hydrolase	CII-EP2	O-Glycan biosynthesis	1.84	3.14	2.01
C_mge-A043-H1-post7-T3	protein	CII-EP2	carbohydrate binding	3.58	3.53	1.65
S_mge-A030-B2-post9-T3	carbonic anhydrase ii	CII-EP2	lyase activity	4.99	4.26	4.89
S_mge-A047-H8-post63-T3	protein	CII-EP2	lyase activity	2.39	2.27	1.70
C_D024-G1_7	carbonic anhydrase xii	CII-EP2	lyase activity	1.57	2.18	3.22
C_D013-C10_75	carbonic anhydrase ii inhibitor	CII-EP2	lyase activity	3.65	3.28	4.11
S_mge-A048-B3-post17-T3	bone morphogenetic proteintype ia	CII-EP2	TGF-beta signaling pathway	2.48	1.99	1.81
C_D019-G3_23	dual specificity phosphatase 7	CII-EP2	MAPK signaling pathway	1.82	2.31	2.64
C_MGE-A027-C11-post82-	dynein light chain 2	CII-EP2	---NA---	2.08	1.95	1.98
C_MGE-A009-H10-57779-T	matrix metalloproteinase 2	CII-EP4	metalloendopeptidase activity	3.88	6.67	1.00
C_mge-A048-D1-post3-T3	tubulin alpha-1 chain	CII-EP4	---NA---	2.32	2.75	1.00
CIII						
C_D027-F7_54	h6 family homeobox 3	CIII-EP1	sequence-specific DNA binding	1.00	1.64	1.00
C_mge-B021-H10-prawn79	cyclin b3	CIII-EP1	Cell cycle	1.00	1.73	1.00
S_MGE-A017-E2-post12-T3	polymerase (dna-directed)delta 4	CIII-EP1	DNA replication	1.00	1.82	1.00
S_MGE-A021-G4-post30-T3	mads box transcription enhancer	CIII-EP1	sequence-specific DNA binding	1.00	1.57	1.00
S_mge-A030-F6-post45-T3	Echinonectin	CIII-EP1	---NA---	1.00	1.56	1.00
C_D021-G5_39	transaldolase 1	CIII-EP2	carbohydrate metabolic process	1.64	1.00	1.00
C_MGE-A022-F4-post29-T	squamous cell carcinoma antigen	CIII-EP2	positive reg. of cell differentiation	1.63	1.00	1.00
C_MGE-A023-A6-post40-T	phosphoenolpyruvate carboxykinase 1	CIII-EP2	aerobic respiration	2.10	1.00	1.00
C_mge-B027-F10-prawn77	Nemo like kinase	CIII-EP2	---NA---	2.06	1.00	1.00
C_mge-B044-C1-prawn2_T	acyl-coenzyme ac-4	CIII-EP2	Fatty acid metabolism	1.53	1.00	1.00
C_mge-B045-B10-prawn73	isocitrate lyase	CIII-EP2	aerobic respiration	1.64	1.00	1.00
S_MGE-A005-D3-19319-T3	transketolase	CIII-EP2	carbohydrate metabolic process	1.51	1.00	1.00
S_MGE-A011-E9-76868-T3	alpha3-fucosyltransferase	CIII-EP2	carbohydrate metabolic process	1.61	1.00	1.00
S_MGE-A023-C10-post74-T3	bone morphogenetic proteintype 1b	CIII-EP2	positive reg. of cell differentiation	1.57	1.00	1.00
S_mge-A030-E5-post36-T3	formin 2	CIII-EP2	Dorso-ventral axis formation	1.50	1.00	1.00
S_mge-A037-A6-post40-T3	modulator of activity of ets genes	CIII-EP2	Dorso-ventral axis formation	2.06	1.00	1.00
S_MGE-A050-D9-post67-T3	aldehyde dehydrogenase	CIII-EP2	carbohydrate metabolic process	1.72	1.00	1.00
C_MGE-A006-D10-28975-T	Galaxin-like	CIII-EP3	---NA---	1.90	1.00	-2.23
C_GS01ZF02	catalase	CIII-EP3-7	response to oxidative stress	1.64	1.00	-2.05

C_MGE-A009-A3-57716-T3	elegans protein	CIII-EP3-7	response to oxidative stress	1.95	1.00	-2.37
C_MGE-A016-A12-post88-	dbh-like 1	CIII-EP3-7	oxidoreductase activity	1.85	1.00	-2.23
C_mge-A041-E6-post44-T	d-amino acid oxidase	CIII-EP3-7	oxidoreductase activity	1.59	1.00	-1.34
C_MGE-B038-C10-prawn74	phytanoyl-dioxygenase	CIII-EP3-7	oxidoreductase activity	1.61	1.00	-2.45
C_MGE-C019-A8-pre56_T3	oxidase peroxidase	CIII-EP3-7	response to oxidative stress	1.62	1.00	-2.25
S_mge-A041-E3-post20-T3	protein	CIII-EP3-7	oxidoreductase activity	2.00	1.00	-2.48
C_GS01MF08	zinc finger protein 198	CIII-EP4	cation transport	1.83	1.63	1.00
C_mge-a013-E4-post28-T	typealpha 3	CIII-EP4	regulation of morphogenesis	1.58	1.64	1.00
C_MGE-A019-G7-post54-T	hepatocyte nuclear factor 4	CIII-EP4	transcription factor activity	1.52	1.57	1.00
C_mge-A042-G6-post46-T	na+ k+alpha 3 polypeptide	CIII-EP4	cation transport	2.03	1.82	1.00
C_MGE-A049-A11-post80-	na+ k+ atpase beta subunit	CIII-EP4	embryonic development ending…	1.80	1.76	1.00
C_mge-C004-D7-pre51_T3	ubiquinol-cytochrome crieske iron-sulfur	CIII-EP4	cation transport	1.75	1.75	1.00
S_mge-a013-F2-post13-T3	splicing factorsubunit 1	CIII-EP4	transcription factor activity	1.00	1.00	1.66
S_MGE-A015-H9-post71-T3	jumonji domain containing 5	CIII-EP4	cation transport	1.57	2.38	1.00
S_MGE-A023-B6-post41-T3	potassium inwardly-rectifying channel	CIII-EP4	cation transport	2.16	1.63	1.00
S_MGE-A027-C3-post18-T3	anion sugar transporter	CIII-EP4	anion transport	1.63	1.73	1.00
S_MGE-A027-C4-post26-T3	ankyrin repeat and btb domain	CIII-EP4	transcription factor activity	2.11	1.78	1.00
S_MGE-A035-E4-post28-T3	na+ k+ atpase alpha subunit	CIII-EP4	embryonic development ending…	1.52	1.84	1.00
S_mge-A048-E6-post44-T3	homeobox protein six3	CIII-EP4	transcription factor activity	2.23	1.51	1.00
S_mge-B027-D1-prawn3_T3	sodium bicarbonate cotransporter	CIII-EP4	anion transport	1.94	1.77	1.00
S_mge-B034-F3-prawn21_T3	na+ k+ atpase alpha subunit	CIII-EP4	embryonic development ending…	1.85	1.80	1.00
C_D045-F12_94	nuclear ribonucleoprotein a b	CIII-EP5	---NA---	1.95	1.66	1.75
S_MGE-A004-H2-H9715-T3	E-selectin	CIII-EP7	---NA---	1.00	1.59	-2.22
CIV						
C_MGE-A015-G5-post38-T	cyan fluorescent protein	CIV-EP1	---NA---	1.00	1.00	6.02
C_D040-B2_10	rbm3 protein	CIV-EP1	translation	1.00	1.00	1.86
C_GS01YC08	40s ribosomal protein s19	CIV-EP1	translation	1.00	1.00	1.71
C_GS01dB04	repressor of e1a-stimulated	CIV-EP1	translation	1.00	1.00	1.95
C_GS01QC09	60s ribosomal protein l12	CIV-EP1	translation	1.00	1.00	1.80
S_GS01XF12.b1.ab1	s15 ribosomal protein	CIV-EP1	translation	1.00	1.00	1.60
C_MGE-A014-D5-POST35-T	ribosomal protein s20	CIV-EP1	translation	1.00	1.00	1.52
S_GS01HA11	ribosomal protein s15	CIV-EP1	translation	1.00	1.00	1.57
C_GS01NC05	ribosomal protein s15	CIV-EP1	translation	1.00	1.00	1.50

C_mge-C002-H2-pre15_T3	ribosomal protein l26	CIV-EP1	translation	1.00	1.00	1.59
S_mge-A048-F1-post5-T3	ribosomal protein s12	CIV-EP1	translation	1.00	1.00	2.39
C_MGE-A016-C3-post18-T	ribosomal protein s12	CIV-EP1	translation	1.00	1.00	1.83
C_mge-C003-E2-pre12_T3	ribosomal protein s24	CIV-EP1	translation	1.00	1.00	1.59
C_GS01ZE08	ribosomal protein l27	CIV-EP1	translation	1.00	1.00	1.60
S_GS01HE04	40s ribosomal protein s19	CIV-EP1	translation	1.00	1.00	1.55
C_MGE-A001-A12-post88-	ribosomal protein s7	CIV-EP1	translation	1.00	1.00	1.55
C_D034-D7_52	ets variant gene 6 (tel oncogene)	CIV-EP1	pattern specification process	1.00	1.00	2.16
C_D009-H8	transcription factor mafb	CIV-EP1	pattern specification process	1.00	1.00	2.16
C_MGE-A031-G1-post6-T3	paired box protein 7	CIV-EP1	pattern specification process	1.00	1.00	20.53
C_MGE-A022-F7-post53-T	sprouty homolog 3	CIV-EP1	pattern specification process	1.00	1.00	1.58
S_mge-B022-G12-prawn_T3	cytoplasmic polyadenylation	CIV-EP1	pattern specification process	1.00	1.00	1.52
C_mge-B035-A4-prawn24_	sonic hedgehog	CIV-EP1	pattern specification process	1.00	1.00	1.93
S_mge-A042-G5-post38-T3	snf1-like kinase	CIV-EP1	regulation of cell differentiation	1.00	1.00	3.41
C_D024-A3_17	transcription factor ighm enhancer 3	CIV-EP1	regulation of cell differentiation	1.00	1.00	2.30
C_MGE-A018-C8-post58-T	activating transcription factor 4	CIV-EP1	regulation of cell differentiation	1.00	1.00	2.29
C_mge-B023-C3-prawn18_	btgmember 2	CIV-EP1	regulation of cell differentiation	1.00	1.00	5.06
S_GS01vA01	beta-catenin	CIV-EP1	Wnt signaling pathway	1.00	1.00	1.58
S_MGE-A023-F2-post13-T3	Notch-like protein	CIV-EP1	Notch signaling pathway	1.00	1.00	1.63
S_mge-A045-D10-post75-T3	snail homolog 1	CIV-EP1	Adherens junction	1.00	1.00	1.81
S_MGE-A017-H8-post63-T3	heat shock protein 70	CIV-EP1	MAPK signaling pathway	1.00	1.00	1.54
C_D006-B2	profilin	CIV-EP1	Regulation of actin cytoskeleton	1.00	1.00	1.56
C_mge-A043-F1-post5-T3	alpha 1	CIV-EP1	Regulation of actin cytoskeleton	1.00	1.00	1.63
C_MGE-A049-H4-post31-T	Actin	CIV-EP1	Regulation of actin cytoskeleton	1.00	1.00	2.37
C_D009-C6	Gelsolin	CIV-EP1	---NA---	1.00	1.00	1.88
S_MGE-A034-B11-post81-T3	syntaxin 6	CIV-EP1	SNARE vesicular transport	1.00	1.00	1.68
C_D009-C9	v-jun sarcoma oncogene-like	CIV-EP2	transcription regulator activity	2.22	1.00	9.13
C_MGE-A012-C12-86590-T	yes-associated protein 1	CIV-EP2	transcription regulator activity	1.72	1.00	3.28
S_MGE-A031-G12-post94-T3	cryptochrome 2	CIV-EP2	response to abiotic stimulus	1.58	1.00	4.08
C_MGE-A017-B1-post1-T3	heat shock transcription factor 1	CIV-EP2	response to abiotic stimulus	1.87	1.00	2.28
S_MGE-A009-G12-57794-T3	cryptochrome 1 (photolyase-like)	CIV-EP2	response to abiotic stimulus	1.64	1.00	2.57
S_mge-A044-B6-post41-T3	fgfr3 protein	CIV-EP3	skeletal system development	1.00	1.54	1.89
C_mge-A045-C3-post18-T	typealpha 1	CIV-EP3	skeletal system development	1.00	2.05	2.19
C_MGE-A012-H12-86595-T	collagen triple helix repeat	CIV-EP3	extracellular matrix part	1.00	1.74	2.93

C_MGE-C019-H12-pre95_T	complement component c3	CIV-EP3	defense response	1.00	1.77	2.50
C_D048-C10_75	ccaat enhancer binding protein	CIV-EP3	defense response	1.00	1.88	2.74
S_mge-A044-B6-post41-T3	Fibroblast growth factor receptor-like	CIV-EP3	Regulation of actin cytoskeleton	1.00	1.54	1.89
C_mge-B027-E12-prawn92	calmodulin2	CIV-EP3	---NA---	1.00	1.57	1.88
C_D011-E7_53	early growth response 1	CIV-EP4	anatomical structure development	1.83	1.66	10.11
S_mge-A038-F4-post29-T3	loc511371 protein	CIV-EP4	anatomical structure development	1.52	1.96	4.03
S_MGE-A049-F10-post77-T3	tyrosine 3-monooxygenase	CIV-EP4	anatomical structure development	1.54	1.53	1.74
S_MGE-A006-B9-28965-T3	ADAM metallopeptidase	CIV-EP5	---NA---	-1.55	-1.79	1.75
C_D045-C10_75	frizzled-related protein	CIV-EP5	---NA---	-1.64	-1.81	1.77
C_D003-B10	n-myc downstream regulated	CIV-EP6	---NA---	-1.57	1.00	1.94
C_MGE-A021-A1-post0-T3	epididymal secretory protein e1	CIV-EP6	---NA---	-1.60	1.00	1.67
C_MGE-B015-F1-prawn5_T	calmodulin3	CIV-EP6	---NA---	-1.64	1.00	1.66

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CHAPTER 6. GENERAL DISCUSSION.

6. 1. Coral metamorphosis and the calicoblastic ectoderm.

Metamorphosis in scleractinian corals is a unique process that differs significantly from that experienced by other anthozoans. During this transition, tissue morphogenesis gives rise to a calcifying epithelium responsible for the secretion of the coral skeleton (Puverel et al., 2005) that forms the basis of coral reefs (Veron, 2000; Gunderson, 2007). Coral metamorphosis involves two major morphological changes 1) an oral endodermal rearrangement that gives rise to the mesenteries and 2) an aboral ectodermal process that generates the calcifying tissue. This latter involves the reorganization of the aboral ectoderm, from a columnar epithelium into a squamous calcifying cell type known as the "calicoblast" (Vandermeulen, 1974; Clode and Marshall, 2004).

In hydrozoans, an interstitial population of undifferentiated cells (I-cells) in a constant mitotic cycle migrates and differentiates into tissue specific cell types during metamorphosis, asexual reproduction and/or regenerative processes (Hyman 1940; Martin 1990; Bode 1996). In the hydroid *Pennaria tiarella,* this I-cell population increases in number during development, reaching its peak at the planula stage (Martin and Archer 1997). The identification of a small rounded cell type whose morphology resembles that of interstitial cells from hydrozoans (Hyman 1940) in all the *Acropora* stages used to originate primary cell cultures (chapter 2), together with the result that cultures originated from planulae gave characteristically higher yields in semi-quantitative PCR using coral specific primers, suggests that an I-cell like population capable of founding cell cultures increases in abundance during the development of coral larvae. I-cells in anthozoans have only been associated to gamete differentiation (Gaino *et al,* 2008), however, all of the cell types derived from I-cells in hydrozoans (gland cells, neurons, nematocysts and gametes; Bode 1996) are present in anthozoans (Hyman 1940). In corals, pluripotent cell populations could also be the precursors of adult specific cell types, including those that appear only after metamorphosis such as calicoblasts and desmocytes.

The results of microarray analysis (chapter 5) can be interpreted with reference to this hypothesis. The up-regulation in both planula and adult polyps of molecules involved in pattern specification processes (GO:0007389) and the regulation of cell differentiation (GO:0045595)(Table 5.2) supports this idea. Furthermore, up-regulation in planulae of molecules belonging to the GO categories: positive regulators of apoptosis (GO:0043065) and larval development (GO:002164) as well as a number of components of conserved signalling pathways known to regulate apoptosis, cell differentiation, migration and proliferation across the Metazoa (Table 5.2), suggest that to some extent morphogenesis of adult structures is initiated at the planula stage. Consistent with this, there is evidence that adult structures such as mesenteries may be formed before settlement in corals (Vandermeulen, 1974; Babcock and Heyward, 1983; Szmant-Froelich *et al.,* 1980).

According to this, is reasonable to think that some adult cell types may arise from partially committed precursors that originate prior to settlement, and that these are able to differentiate into adult specific cell types during metamorphosis, asexual reproduction, growth and regeneration as is the case in hydrozoans. The observed up-regulation in planulae and adult polyps of molecules whose orthologs induce chondrocyte differentiation in mammals (chapter 5) is consistent with the idea that calicoblast precursors originate in *Acropora* planulae prior to metamorphosis. On the other hand, the finding that a coral specific CaM-like molecule that interacts *in vitro* with *Acropora* CaM (chapter 4), alpha type 1 collagen - one of the major structural components of bone (Ge and Greenspan, 2006) - and an FGF receptor (FGF signaling mediates osteogenic differentiation in mammals; Selfors *et al.,* 1998; Ng *et al.,* 2007) were up-regulated in primary and adult polyps suggests that calcium signaling might regulate the final stages of calicoblast differentiation in *Acropora*.

The finding that a number of EF-hand calcium sensors such as calmodulin, calumenin, calbindin, calreticulin and calcineurin (Table 5.2) were up-regulated in planulae indicates that calcium signaling is a key regulator of planula cellular activity and might be involved in the signaling transduction mechanisms leading to settlement and metamorphosis. Despite this, the extent at which cellular differentiation mechanisms in corals resemble those described in better characterised model

organisms and the nature of coral stem cells remain unclear. More research is needed to test and validate these observations.

6. 2. Coral calcification and the organic matrix.

After settlement, juvenile polyps secrete a species-specific corallite that constitutes a precursor to colony morphology. The structure of the primary corallite has been used since the 19th century for taxonomic purposes as its structure reflects the major scleractinian phylogenetic lineages (Le Tissier, 1988; Vandermeuden and Watabe, 1973; Cuif and Dauphin, 2005). Juvenile skeletons differ from adult skeletons in both structure and crystal type. While juvenile skeletons are lightly mineralised with smaller random orientated crystals of both calcite and aragonite, adult skeletons display a fibrous parallel growth that allows massive aragonite calcification (Vandermeuden and Watabe, 1973).

Two different calcifying regions give rise to the juvenile skeleton. The first of these is the base of the polyp, which is responsible for the deposition of the first skeletal elements to be formed – the basal plate and protosepta – and these contain both calcite and aragonite crystals. The second calcifying region is the mesenteries. These are responsible for the massive parallel aragonite growth characteristic of adult calcification (Le Tissier, 1988; Vandermeuden, 1974; Vandermeuden and Watabe, 1973). The latter type of calcification is evident 72 hours after settlement (Vandermeuden and Watabe, 1973; Vandermeuden, 1975; Le Tissier, 1988; Cuift and Dauphin, 2005) and is consistent with the observed timing of mesenteries formation in *Acropora* juvenile polyps.

The observation of three galaxin related molecules with distinct but partially overlapping spatial and temporal expression patterns led us to suggest roles for this family of proteins during basal plate deposition and mesenterial calcification (chapter 3). While both of the Amgalaxin-like genes are expressed exclusively in the early stages of calcification and are likely to be involved in basal plate formation (Fig 3.4 and 3.5), Amgalaxin is expressed in the mesenteries (Fig 3.6) and continues to be expressed in the adult (chapter 3). Microarray results (chapter 5) also show up-

regulation of Amgalaxin-like1 in the flat polyp stage (i.e. when the basal plate is formed), and are thus consistent with the in situ and virtual northern results for this gene. Likewise, the observed up-regulation of homologs of the ECM proteins echinonectin and E-selectin (table 5.2) in primary polyps is consistent with the idea that differential expression of organic matrix components is responsible for determining crystal polymorph and growth during *Acropora* skeletogenesis. Functional assays are necessary to understand the roles of these secreted proteins.

The up-regulation in all calcifying stages (CII) of transcripts involved in O-Glycan biosynthesis (GO:0004553), metalloendopeptidase activity (GO: 0004222) and regulators of the tubulin-cytoskeleton (Table 5.2) is consistent with the idea that matrix biosynthesis rather than $CaCO_3$ deposition is the limiting factor controlling coral skeleton deposition (Allemand *et al*., 1998). In a similar way, up-regulation in calcifying stages of molecules involved in O-glycosylation (GO:0004553) is consistent with the observation that glycosylation is a prerequisite for skeleton formation in corals (Allemand *et al*. 1998) as it is in echinoderms (Ameye *et al*., 2001),

The fact that Amgalaxin-like1 expression was initially restricted to columnar ectodermal cells located in the aboral end of planulae, which according to Le Tissier (1988) is the last region to differentiate into calicoblastic cells, suggest that Amgalaxin-like1 expression is not restricted to calicoblastic cells and lead us to suggest that these putative secretory columnar transectodermal cells are involved in the initial planula attachment during settlement and metamorphosis. Clode and Marshall (2004) found that levels of intracellular calcium were lowest in this same region - at the aboral end of planula - in *Pocillopora damicornis*. Whether there is a functional relationship between Amgalaxin-like1 expression in planulae and the low intracellular calcium concentration reported in *Pocillopora* larvae remains to be investigated.

Finally, the observed up-regulation in *Acropora* planulae of molecules involved in lipid metabolism and phospholipid biosynthesis (Table 5.2) might not only reflect larval use of stored lipids as energy sources (Vandermeuden, 1974) but could also represent a mechanism by which calcium ions are delivered to the calcification site

prior to skeleton deposition. Acidic phospholipids are products of lipid metabolism that are known to promote the deposition of calcium salts (Kant *et al.* 1996) and there is evidence that calcium is accumulated in endodermal lipid containing vesicles in both planula and settled (aposymbiotic) coral larvae (Clode and Marshall, 2004). Several authors have suggested that acidic phospholipids comprise the calcium-binding fraction in the calcifying OM of hermatypic corals (Isa and Okasaki, 1987; Watanabe *et al.,* 2003). More research is necessary to characterize organic matrix components and their respective function during both juvenile and adult *Acropora* skeletogenesis.

6. 3. Final remarks and future outlook.

Although scleractinian corals are responsible for the underlying framework of coral reefs (Veron, 2000; Gunderson, 2007), the molecular mechanisms regulating coral calcification are still poorly understood. The results reported here focused on early stages of *Acropora* calcification and suggest high levels of complexity in the mechanisms regulating coral skeletogenesis. Calcification requires the coordination of multiple signaling pathways involved in the transport of calcium and carbonate ions as well as the synthesis and transport of OM precursors. Furthermore, as the initiation of calcification correlates with the aboral ectodermal rearrangement that gives rise to the calicoblastic ectoderm, the cellular mechanisms regulating this transition are of great importance in understanding the biology of the calcifying cell.

The results presented here indicate the importance of characterising larval cell population and the possible roles of pluripotent cell population as precursors of adult cell types including the calicoblast. Likewise, the role of calcium as an intracellular second messenger and the molecules involved in translating its signal are of vital importance in understanding the regulatory gene networks underlying skeleton deposition and metamorphosis in *Acropora*. As shown in this study, microarray studies represent a good tool to generate hypotheses in many areas of coral biology, however the development of these ideas is often restricted because conceptual models are of necessity based on analogous pathways in model organisms.

Functional and physiological assays are necessary to elucidate the cellular localization and physiological function of the *Acropora* molecules characterized in this study as well as the roles of coral specific molecules identified by microarray analysis. This can be achieved via cellular assays using coral cell lines, microinjection, and RNA interference methodologies. Morpholinos have been used to knock down genes during early stages of development (Rentzsch et al., 2008; Magie et al., 2007) and transgenic *Hydra* has been generated by microinjection of early embryos (Khalturin et al., 2007). Similar approaches could be used in coral larva to study the function and localization of genes of interest. Further studies on coral calcification will not only provide valuable information for the management and conservation of coral reefs but also due to the basal nature of Scleractinia among metazoans, it will also contribute to the understanding of the evolution of calcium dependent cellular pathways in the animal kingdom.

6. 4. References.

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