UNIVERSITY OF DERBY

DEVELOPING PROTOCOLS AND METHODS TO PREDICTABLY INDUCE *EX SITU* BROADCAST CORAL SPAWNING AND INCREASE POST SETTLEMENT SURVIVORSHIP

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Doctor of Philosophy 2019

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Contents

Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 8

Appendices - Tables and Figures

Abstract

The production of broadcast spawning gamete material *ex situ* has great potential in developing areas for coral research and/or to support initiatives aimed at rebuilding damaged reefs utilising sexually produced spat. Current effectiveness of such restoration practises are limited due to the high mortality rates post settlement and therefore methods aimed at increasing survival, and therefore productivity, are required and vital in order to further support upscaling of such practices. Therefore, this thesis focuses on developing methodology to predictably induce broadcast reef building corals to spawn *ex situ* and investigate ways to maximise post settlement survivorship.

Acquisition of broodstock for any *ex situ* breeding project is essential. Chapter two describes the methodology designed and implemented in order for me to carry out long distance transportation (a journey time of ~34 hrs) of large (16-37 cm) gravid *Acropora hyacinthus* (Dana, 1846) colonies from fringing reefs south of Singapore to the Horniman Museum and Gardens, London. Collection was purposefully timed to occur just before the predicted annual mass spawning event and on the day of transportation 12 of the 14 genotypes contained large visible oocytes, which spawned *ex situ* within the same lunar month as those in the wild.

A closed system mesocosm aquarium was designed at the same time, as described in chapter 3 that utilises microprocessor technology to accurately replicate environmental conditions associated with stimulating broadcast spawning events (photoperiod, seasonal solar irradiance, lunar cycles and seasonal temperature) from two synchronous spawning locations, Singapore and the Great Barrier Reef, Australia. Coupled with appropriate coral husbandry, four species (*A. hyacinthus*, *Acropora millepora* (Ehrenberg 1834), *Acropora tenuis* (Dana 1846) and *Acropora microclados* (Ehrenberg 1834)) completed full gametogenic cycles and spawned in a fully closed artificial *ex situ* environment (in synchrony with the wild).

The effects of spawning broadcast corals *ex situ* is currently unknown, therefore following gamete release embryological development stages of three acroporids (*A. millepora*, *A. tenuis* and *Acropora anthocercis* (Brook 1893)) was assessed utilising scanning electron microscopy and confocal laser scanning microscopy techniques (Chapter 4). No abnormal developmental effects (as result of the *ex situ* environment) were observed, but the study built on previous works to provide increased detail of fertilisation and early cell stages.

Reef building corals typically undergo a type III survival curve in their early life stages, with high mortality rates during early ontogeny. Increased post settlement survival can occur due to

size mediated multi-genotype settlement aggregations and species hybridisation. These two factors were empirically tested (Chapter 5) in pure and interspecific hybrid crosses of *A. millepora* and *Acropora anthocercis*. Increased survival, and to a lesser extent growth, were observed in post settlement entities with >2 genotypes compared with single genotype primary polyps and in interspecific hybrid crosses compared to pure species crosses, highlighting the role of hybridisation vigour.

Reef herbivory may enhance coral settlement and recruitment success however the influence of herbivory size classes on survival benefits are not ubiquitous. In order to assess the positive role that microherbivory may contribute to maximising coral survival and growth *ex situ* two species, the Tuxedo sea urchin, *Mespilia globulus* (Linnaeus, 1758) and the reef building coral, *A. millepora*, were co-cultured at varying densities. Increasing density of microherbivory significantly enhanced coral survival and growth, highlighting this as a potentially beneficial practise in improving productivity of coral produced via sexual reproduction.

Finally closing the life cycle of a target organism marks an important milestone in any *ex situ* breeding programme or aquaculture method. Chapter 7 describes the production of the first F2 generation of *A. millepora* in a fully closed aquarium environment.

In summary, it is therefore hoped this thesis will, in part, make a contribution to coral sexual reproductive research and the important work of reef restoration, particularly in light of the global decline in coral reef ecosystems.

Preface

The work, including conceptual design of experiments, data collection and writing within this thesis, has been solely authored by the doctoral candidate with guidance from the supervisory team. Guidance for the statistical analysis and R coding was provided by Dr Mark Bulling, Dr Alfred Burian and Dr Adriana Humanes.

Histological preparations within chapter 2 and appendix 1-3, were conducted by International Zoo Veterinary Group.

Inductively coupled plasma atomic emission spectroscopy (ICP-OES) water chemistry analysis in chapter 3 (appendix 4 & 5) were conducted by Triton Applied Reef Biosciences.

Any images used that were not taken by the candidate have been credited appropriately with their permission.

Where chapters have been published the doctoral candidate has been the lead author, preparing the manuscript, with only guidance from co-authors.

All ethical consideration were discussed and approved during annual ethical review meetings at the Horniman Museum and Gardens.

The work contain within this thesis has been disseminated through a number of channels, list here;

Publications;

Craggs, J. R. K., Guest, J. R., Bulling, M., & Sweet, M. (2019). Ex situ co culturing of the sea urchin, *Mespilia globulus* and the coral *Acropora millepora* enhances early post-settlement survivorship. *Scientific Reports*, (August), 1–12. https://doi.org/10.1038/s41598-019-49447-9

Craggs J., Guest J.R., Brett A., Davis M., Sweet M. (2018) Maintaining spawning timing in *Acropora* corals following long distance inter-continental transportation. Journal of Zoo and Aquarium Research 6(2) 30-36 https://doi.org/10.19227/jzar.v6i2.317

Craggs J, Guest JR, Davis M, Simmons J, Dashti E, Sweet M. (2017) Inducing broadcast coral spawning *ex situ*: Closed system mesocosm design and husbandry protocol. Ecol Evol; 7:11066–11078. http://doi.org/10.1002/ece3.3538

Conferences;

Craggs, J., Guest, J., Bulling, M. & Sweet, M (2018) *Ex situ* co-culturing of the sea urchin, *Mespilia globulus* and the coral *Acropora millepora* enhances early post-settlement survivorship: implications for large scale coral propagation. Reef Conservation UK; ZSL London Zoo, December.

Craggs, J., Davis, M., O'Neil, K., Graves, S., Guest, J. & Sweet, M (2018) A synopsis of three years of *ex situ* broadcast spawning research and *in situ* goals for the future. European Union of Aquarium Curators; The Deep, Hull, October.

Craggs, J., O'Neil, K., Graves, S., Guest, J. & Sweet, M (2018) Developing land-based coral facilities to stimulate multiple *ex situ* broadcast spawning events per year for reef restoration; Regional Aquatic Workshop, Florida Aquarium, May.

Craggs, J., Guest, J. & Sweet, M (2018) *Ex situ* co-culturing of the sea urchin *Mespilia globulus* and the coral *Acropora millepora* enhances early post settlement survivorship: implications for upscaling reef restoration ambitions; Environmental Sustainability Research Centre annual conference, University of Derby, May.

Craggs, J (2018) Developing *ex situ* protocols to induce multiple broadcast coral spawning events for research and reef restoration, Palau International Coral Research Center, March.

Craggs, J (2018) Developing *ex situ* protocols to induce multiple broadcast coral spawning events for research and reef restoration', London Ocean Group, UCL, January.

Craggs, J., Guest, J., Ball, A. & Sweet, M (2017) Using SEM to map embryogenesis in three reef building hard coral species following captive induced gametogenic cycles and subsequent broadcast spawning events. The Society of Electron Microscope Technology, Natural History Museum, December

Craggs, J., Davis, M., O'Neil, K., Graves, S., Guest, J. & Sweet, M (2017) Developing Land-based coral facilities to stimulate multiple *ex situ* broadcast spawning events per year for reef restoration. European Coral Reef Symposium, Oxford University December.

Craggs, J (2017) Developing protocols for predictable broadcast coral spawning in captivity; Reef Stock X, Denver, Colorado, Feb.

Craggs, J (2017) Developing protocols for predictable broadcast coral spawning in captivity; EcoTech Marine trade conference, UK, March.

Craggs, J., Guest, J. & Sweet, M (2016) Developing protocols for predictable broadcast coral spawning in captivity; International Aquarium Congress, Vancouver Aquarium Marine Science Centre, http://iac2016.venuewest.com/, Sept.

Craggs, J., Guest, J. & Sweet, M (2016) Developing protocols for predictable broadcast coral spawning in captivity; Reef Conservation UK, Zoological Society of London - London Zoo, Dec.

Poster;

Craggs, J., Guest, J., Davis, M., Simmons, J., Dashti, E & Sweet, M (2017) Inducing broadcast coral spawning *ex situ*: Closed system mesocosm design and husbandry protocol. Environmental Sustainability Research Conference, University of Derby, June 2017.

Public outreach and press;

Interview (2018) - The Marine Diaries conducted an interview about Project Coral and my research as part of their Marine Diaries TV series. https://www.youtube.com/watch?v=S6_VYIsbYSU April 2018

Public outreach (2018) - participated in Blue Planet II Late at Natural History Museum joining producers and researchers to host a range of talks, activities and interactive experiences. January.

Interview (2018) - Worldhacks, BBC World Service Can We Save Coral?, January.

Interview (2017) - European Coral Reef Symposium, Oxford University gave an interview with Guardian environment editor - Guardian December.

Natural History Museum (2017), NHM as part of Science Uncovered, an event which is part of the European Researchers' Night, funded by the European Commission http://ec.europa.eu/research/researchersnight/about_en.htm September.

A joint press release between Horniman and Derby University (2017) on first published paper was picked up by the Express in December

Awards;

(2018) Marine Aquarium Societies of North America Aquarist of the Year Award, Las Vegas, Sept.

(2018) Best of RAW; Regional Aquatic Workshop, Florida Aquarium, Florida, May.

(2017) International Society of Reef Studies sponsored student prize for best presentations at European Coral Reef Symposium, Oxford University, Dec.

Acknowledgements

Since completing my undergraduate degree over 20 years ago I had often thought about studying for a PhD and yet I continually questioned my ability to take on such an undertaking. I would therefore like to thank my friend and director of studies Dr Michael Sweet for giving me this incredible opportunity and for believing that I was capable of completing these studies. Your effortless ability to solve problems, provide a reassuring voice and continued encouragement has been paramount. Also a special thanks to my 'roomie' Dr James Guest for agreeing to be my external supervisor. Your knowledge on coral reproduction and restoration practises have long inspired me and also your calm approach, ability to see the bigger picture and to critically evaluate the impact of work has been fantastic. I'm hopeful the that three of us will continue to work together and make changes for the better to reef environments and for the people that depend on them.

For me, the most daunting aspect of the research was always going to be the statistics. Thankfully things have changed a huge amount since I finished my undergraduate degree, with the majority of statistical work now being conducted on the R platform. A massive thanks to my other supervisor, Dr Mark Bulling and to Dr Alfred Burien for patiently getting my brain into gear again, for putting up with my rudimentary statistical / coding questions and generally guiding me through the statistics in this thesis. Dare I say it; I have actually enjoyed this aspect of the research! To quantitatively assess data and pull out key findings has been a really rewarding process and I feel that I have grown as a scientist over the three years and look forward to applying my new-found interest in R coding in the future!

It is really important for me to thank so many industry partners, without whose support this work would not have been possible. They have understood the potential of spawning corals *ex situ* from the beginning and without hesitation have provided equipment, chemicals and ICP testing that enable the mesocosms at the Horniman Museum and Gardens to run so seamlessly. Ehsan and Linda Dashti at Triton Applied Reef Biosciences, Justin Lawyer, Patrick Clasen, Chris Clough and Jay Sperandio at EcoTech Marine, along with Jeremy Simmons at Xodus and David Saxby and Stuart Bertram at D&D The Aquarium Solution. You are all awesome, thank you! Huge thanks to Vince Thomas, Aquarium Connections for your amazing build quality and troubleshooting through long-term issues.

Developing the methodology to spawn corals *ex situ* relies on knowledge of how these animals behave in the wild. Therefore I would really like to thank Dr Craig Humphrey at the National Sea Simulator, Australian Institute of Marine Science and Dr Karenne Tun at the National Parks Board, Singapore for sharing their insights and wealth of knowledge of coral reproductive biology and spawning periodicity on the GBR and Singapore. To this end I must also thank my friend Dr Dirk Petersen, SECORE International, whose work has inspired me over the years. I have been lucky enough to attend a number of SECORE workshops and witnessing broadcast coral spawning in the field helped to understand the environmental conditions that stimulate these annual reproductive events. These workshops also led to something more; a global network of amazing scientists and aquarists who I now regard as good friends. Aaron Brett is among those people and I'd like to thank him for being so keen to collaborate on the project shipping broodstock coral from Singapore to the UK. The transportation success was because of his dedication and hard work, particularly liaising with the Singaporean authorities on the CITES paper work.

Thanks to Lyle Squire Jr and Laura Simmons at Cairns Marine for working so closely with me and supplying the corals needed for the experimental work from the Great Barrier Reef. I'm really looking forward to continuing our working relationship in the future.
Shipping the corals from Australia and Singapore required logistical coordination and knowledge of the CITES framework and thanks go to Derek Thompson at the Tropical Marine Centre for patiently overseeing this key part of the research.

Thank you to Scott Graves former director of the Centre for Conservation (CFC), Florida Aquarium and Kerry O'Neil (CFC) for the exciting partnership that we are working on. It's fantastic to see the research from this thesis being applied in Florida for real-world impact. I think the work that is happening in CFC is going to pave a new way forward for reef restoration, utilising land-based coral spawning nurseries as production units for sexual recruits. Exciting times ahead!

I would like to thank Dr Ken Johnson and Dr Alex Ball at the Natural History Museum, London for supporting my Scientific Associate application and guiding me through the principles of scanning electron microscopy and confocal laser scanning microscopy in the amazing facilities within the imaging department of the museum. Documenting developmental stages of animals

through photography has been a strong passion of mine for a number of years and having the opportunity to use these facilities has been an incredibly rewarding part of my studies.

Also special thanks to the Horniman Museum and Gardens' senior management team for allowing me to develop the aquarium and particularly the research focus of the department over the past 10 years. I genuinely believe that a core focus of managing a living collection must be to continue to develop knowledge and husbandry skills for the animals within our care so that we can ultimately support, whether directly or indirectly, species or habitat conservation *in situ*. It is with this in mind that the senior management team members have allowed me to undertake these studies. I hope the advances that I've made here will lead to the positive impact *in situ* that is envisioned.

This work, however, couldn't happen without a fantastic team. With that in mind it is so important for me to thank, both past and present, members of my aquarium team who have been instrumental in developing the research ambition of the department. Michelle Davies, Jem Simmons, James Robson, Dave Gawith, Chloe Priestland and Chris Moocarme have all been part of the small, four person team. Their daily commitment has been monumental. We often say that running a public aquarium as well as developing novel and impactful ideas can only happen when a solid platform is there. That platform only exists because of the often repetitive and manual tasks, coupled with an intuitive understanding of animal biology, water chemistry, water dynamics and plumbing! Without that continuous and sustained level of work there is no solid foundation and therefore no potential. In the past six years we have successfully spawned 21 species of broadcast coral. That is genuinely amazing for any research organisation, let alone from a team of four in a tiny public aquarium. Hats off to you all!

Finally, and most importantly, the person I need to thank the most is my amazing wife, Kate. You have continuously supported me over the past three years in completing this crazy undertaking and I would like to dedicate this dissertation to you. Studying for a PhD full-time whilst working full-time and having a young family has created huge amounts of stress, sleepless nights and a feeling that there is never enough time in the day. Your unwavering support, encouragement and belief in me has been amazing and I could not have done it without you. I know that at times you have felt like a single parent to our amazing boys. It is very much your time to shine and my time to cook! I love you with all my heart.

Chapter 1: Introduction

1.1 Coral reef status

Shallow coral reefs cover an estimated area of 284,300 km^2, an area of less than 1.2 percent of the world's continental shelves and only 0.09% of the total world oceans (Spalding, Ravilious and Green, 2002). They are the most biologically diverse ocean ecosystem, providing refuge to one third of all marine species (Plaisance *et al.*, 2011). This diversity is underpinned by the three dimensional structure formed by calcification and growth of scleractinian reef building corals (Alvarez-Filip *et al.*, 2011; Harrison, 2011). Under conducive conditions coral calcification rates exceed bio erosion factors, such as grazing or boring algal species (Chazottes et al, 1995; Tribollet *et al.*, 2002), resulting in positive net aragonite accretion and over the last thousands of years this has resulted in the largest living ocean structures on earth, including the Great Barrier Reef, Australia.

In addition to the biological importance, the rich diversity of these ecosystems are significant from a human perspective too. Globally an estimated 500 million people (Hoegh-Guldberg *et al.*, 2017) rely on coral reefs, where they provide a valuable source of protein from fishing (Moberg and Folke, 1999) and produce an annual livelihood of an estimated US$36 billion from tourism related activities (Spalding *et al.*, 2017). Reefs also form a highly effective protective barrier to tropical coastlines, diffusing wave energy and preventing coastal erosion during hurricanes, storms and typhoons (Elliff and Silva, 2017). The combined benefits of these ecosystem services throughout the US territories alone are worth an estimated US$3.4 billion per year (Cesar, Burke and Pet-soede, 2003) and worldwide contribute to over US$1 trillion per year to the global economy (Heron *et al.*, 2017).

However, biodiversity of coral reefs is declining. A result of multiple anthropogenic factors (Pandolfi *et al.*, 2003; Carpenter *et al.*, 2008) ranging from local impacts associated with overfishing (Bellwood *et al.*, 2004); pollution (Fabricius, 2005); sedimentation (Richmond, 1993); and global pressures of climate driven ocean acidification (Hoegh-Guldberg *et al.*, 2007; Pandolfi *et al.*, 2011) or thermal stress events leading to widespread coral bleaching and disease (Brown, 1997; Hughes *et al.*, 2017). Since the 1980s, three pan global mass coral bleaching events have occurred; in 1998, 2002 and more recently in 2015-16, in which >80% of corals on the north section of the Great Barrier Reef experienced extreme bleaching and mortality

(Hughes *et al.*, 2017). As a result it is estimated that 30% of reefs are damaged beyond recovery, with 60% more likely to be negatively impacted by some form of human activity by 2030 (Hughes, 2003). This global trend of coral decline and a reduction in key herbivorous species has caused phase shifting in many systems from coral to macroalgae dominated communities (Hughes, 1994; Bellwood *et al.*, 2004).

Reef building corals are regarded as ecological keystone species and are fundamental to ecosystem function. A decline in their population therefore results in a disproportionate loss of associated biodiversity. Like other sessile marine invertebrates, coral populations require successful reproduction and recruitment into a system for the species to persist (Hughes, 2003) but also, to ensure associated biodiversity over time.

Reproduction (incorporating gamete maturation, fertilisation and embryological development forming a planula larvae) is the process in which new individuals are formed. Recruitment is the mechanism by which those individuals, following pelagic dispersal, form part of the coral reef community. While short term recruitment of larvae is directly related to the level of fecundity of the seed adult population (Hughes *et al.*, 2000), long term recruitment may still fail due to a host of factors adversely influencing post settlement survivorship. These include early post settlement mortality due to competitive benthic interactions (Kuffner *et al.*, 2006; Arnold, Steneck and Mumby, 2010), sedimentation (Te, 1992; Jones, Ricardo and Negri, 2015; Fourney and Figueiredo, 2017), predation (Penin *et al.*, 2011; Trapon *et al.*, 2013), disease (Cooper *et al.*, 2007) and/or temperature induced mortality (Nozawa and Harrison, 2007). The study of coral *reproduction* and factors of long-term *recruitment* is therefore of paramount importance if prediction of temporal and spatial coral reef trends are to be drawn (Jones, Ricardo and Negri, 2015).

1.2 Scleractinian reproduction

Scleractinian corals exhibit a broad range of reproductive strategies, including asexual and sexual reproduction (Baird, Guest and Willis, 2009; Harrison, 2011).

1.3 Asexual reproduction

When discussing asexual reproduction it is perhaps useful to define the difference between growth and reproduction.

During growth, polyp numbers increase asexually, either through the division of individual polyps, a process known as intra-tentacular budding (Fig 1.1 A), or the formations of new polyps that grow from the coenosarc interconnecting tissue between existing polyps i.e. extra-tentacular budding (Fig 1.1 B), (Matthai, 1948; Veron, 2000). The net effect is an increase in the number of polyps and overall colony growth. Asexual reproduction on the other hand is the process by which new colonial entities are formed but which share the same genes as the donor colony. Branching and thin plating species in the families Pocilloporidae and Acroporidae (Veron, 2000) are easily fragmented during storms, through fish feeding or other forms of physical disturbance (Highsmith, 1982). The fragmentation of these species is part of their life history strategy, as these asexually produced fragments are able to reattach to the benthos, increasing the number of colonies of that species securing its position within the reef population.

Another strategy, rarely discussed and probably less well understood, is when individual polyps become detached from the parent colony and drift a short distance, reattaching and forming a new colony. During this process, called 'polyp bailout', only the soft tissue of the polyp detaches and a new skeleton forms post settlement. This has been described in the branching coral *Seriapora hystrix* (Sammarco, 1982) and is regularly observed in *Goniopora stokes*i under aquarium conditions (Fig 1.1 C).

A common process in *Fungia* species is individual polyp budding and detachment, however in contrast to polyp bailout daughter polyps that are produced have both soft polyp and skeletal material already formed (Fig 1.1 D & E).

Planula larvae from brooding coral species may also be produced both sexually and asexually (Ayre and Resing, 1986). The brooding corals *Pocillipora damicornis* (Stoddart, 1983) (Fig 1.1 F) and *Favia fragum* (Brazeau, Gleason and Morgan, 1998) (Fig 1.1G) for example, have been shown to even have asexual forms through self-fertilisation and parthenogenesis of unfertilised oocytes. This formation of asexual larvae increases the dispersal potential of genetic material through ocean currents. In addition asexual clonal propagation can occur as early as 2 hrs post fertilisation in scleractinian corals (Heyward and Negri, 2012). During this early period of ontogeny the developing embryos are not motile (behave passively), being moved by wind and ocean currents. At this delicate stage of development the sheering forces of wave action may break the dividing blastomeres, which continue development to form viable planula larvae. This mode of asexual reproduction further increases the dispersal mechanisms. However, a drawback to asexual reproduction is limited genetic variation, which can result in constrained adaption ability during environmentally stressful periods such as El Niño induced bleaching (Baums, 2008).

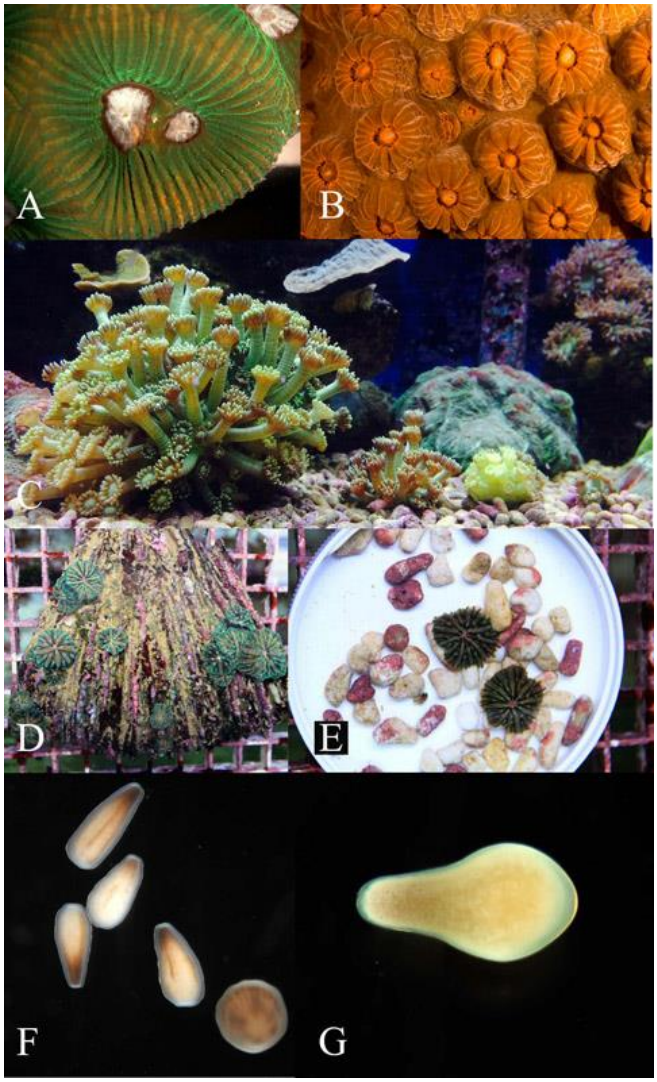


Figure 1. 1 Modes of asexual growth and reproduction in scleractinian reef building corals.

(A) *Goniastrea palauensis* polyp dividing via intra-tentacular budding; (B) New polyps forming from the coenosarc tissue via extra-tentacular budding on *Cyphastrea serailia*; (C) New *Goniopora stokesi* colonies forming via polyp bailout; (D) Daughter polyps growing from an old *Fungia spp*. skeleton; (E) Daughter *Fungia spp.* polyps recently detached from parent polyp; (F) *Pocillopora damicornis* & (G) *Favia fragum* planula larvae produced in an aquarium via asexual self-fertilisation.

All images taken by J Craggs

1.4 Sexual reproduction

In contrast to the limited genetic variability of asexual division, sexual reproduction involves the production of new genetic diversity through two mechanisms; chromosomal crossover during meiosis I within each parent and the contribution of two haploid gametes (oocyte and sperm) from each parent forming the diploid zygote during fertilisation (Fadlallah, 1983). It is therefore through sexual reproduction that increased genetic diversity occurs in a population and gives rise to potentially beneficial adaptations, such as increased thermal tolerance during bleaching events (Willis *et al.*, 2006; van Oppen *et al.*, 2015).

Scleractinian corals express four sexual reproductive characteristics; hermaphroditic broadcast spawners, gonochoristic broadcast spawners, hermaphroditic brooders, and gonochoristic brooders (Harrison, 2011). The reproductive ecology of broadcast spawners are expressed by large colony size and short reproductive periods, whereas brooding species are typically smaller with extended planulation periods (Szmant, 1986).

Species in which polyps or colonies have separate sexes are termed gonochoristic, those that contain both male and female reproductive gonads are hermaphrodites. Most hermaphrodites are simultaneous, with colonies containing both ovaries and spermatophores at the same time (e.g most *Acropora*, faviids and some pocilloporids) (Richmond, 1997), however some species are known to be sequential hermaphrodites (Rinkevich, 1987; Guest *et al.*, 2012). Of the 444 species of scleractinian corals for which sexual reproductive information is available (Babcock *et al.*, 1986; Baird, Guest and Willis, 2009) broadcast spawning is the dominant mode comprising of some 85% of species, with the remaining 15% of species being brooders.

Irrespective of the mode, the onset of sexual maturity is determined by colony age and size (Iwao *et al.*, 2010; Baria *et al.*, 2012). Production of gametes requires a high level of energy investment by the coral and marked reductions in the ratio of energy content in reproductive somatic tissues (gonadosomatic index) have been noted between pre and post spawning tissues (Leuzinger, Anthony and Willis, 2003). Resource partitioning through autotrophic, and possibly heterotrophic inputs, are likely play a role in gamete development and the mechanism of this resource allocation may well differ between brooding vs broadcast spawning species due to periodicity of the gamete production and release.

1.4.1 Brooders

In brooding coral species fertilisation and subsequent embryogenesis occurs internally within the polyps (Pires, Castro and Ratoo, 2000) (Fig 1.2 A). Following embryogenesis free-swimming planula larvae are released into the water column, where they settle to metamorphose into a polyp (Golbuu and Richmond, 2007). Planulation can occur throughout the year with peak outputs linked to the lunar cycle (Richmond and Jokiel, 1984; Jokiel, Ito and Liu, 1985) and their reproductive strategy is characterised by the production of fewer but more frequently released larvae. Most brooders exhibit vertical transmission of Symbiodiniaceae, transferring symbionts from maternal parent directly to the planula (Thornhill, Fitt and Schmidt, 2006; Stat *et al.*, 2008) (Fig 1.2 B). In many species newly settled primary polyps contain green fluorescent proteins (GFP) (Roth, Fan and Deheyn, 2013; Grinblat *et al.*, 2018), which under the correct spectrum of lighting can be detected in darkness (Mazel, 1995) (Fig 1.2 C&D). The development of fluorescent imaging techniques has enabled more accurate recruitment studies to be performed through fluorescent censusing by enabling early cryptic recruits and numbers to be identified with greater ease (Baird, Salih and Trevor-Jones, 2006; Hsu *et al.*, 2014).

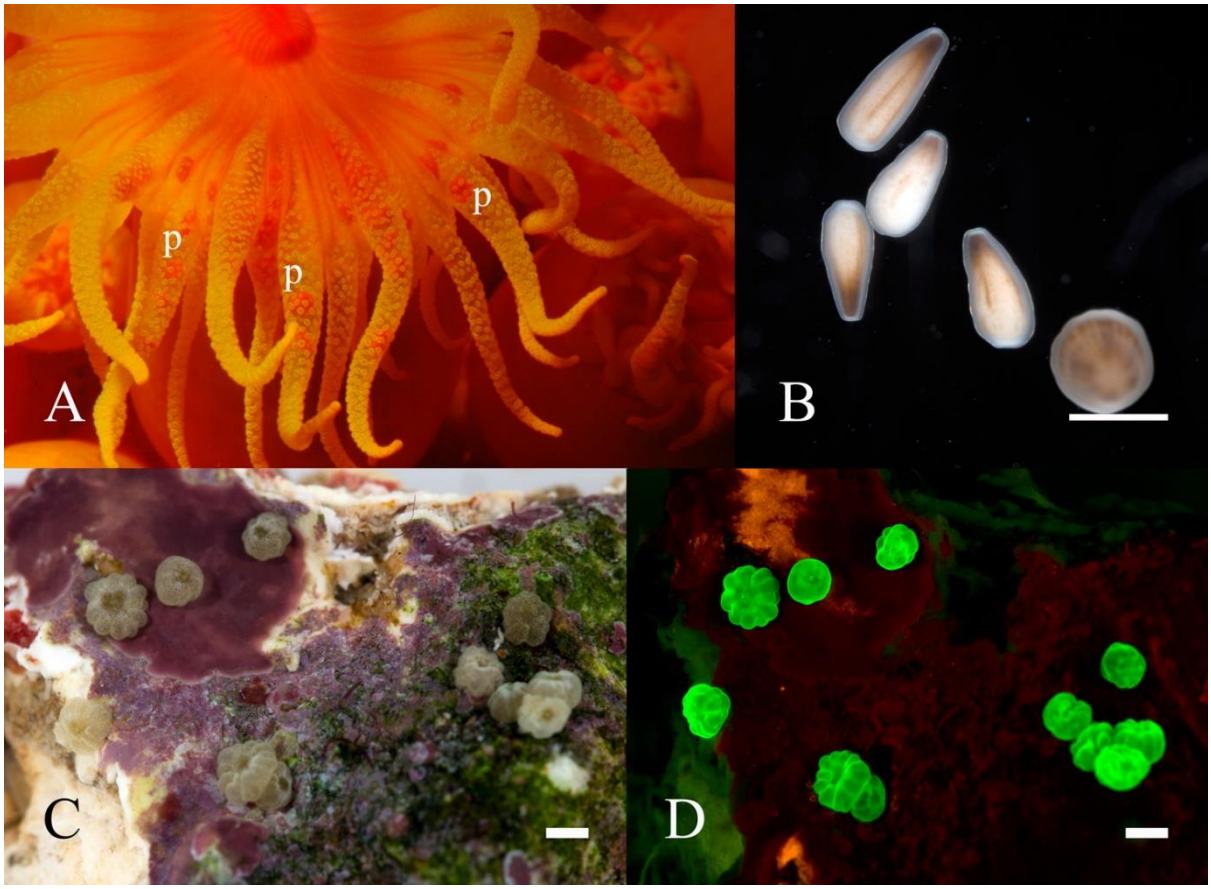


Figure 1. 2 Gamete development, planulation and early post settlement in brooding coral species.

(A) Developing planulae (p) within the tentacles of *Tubastraea coccinea*; (B) Newly released *Pocillopora damicornis* planula containing Symbiodiniaceae (zooxanthellae) via vertical transmission from maternal parent; (C) One day post settled *Favia fragum* primary polyps under full spectrum light; (D) The same *Favia fragum* primary polyps as (C) imaged under Royal Blue – excitation 440 – 460 nm, emission 500 nm longpass (NightSea, BlueStar Flashlight) highlighting Green Fluorescent Proteins (GFP). Scale 1 mm.

All images taken by J Craggs

1.4.2 Broadcast spawners

In contrast to brooding species, broadcast spawning corals release gametes into the water (Fig 1.3 A, Fig 1.4 A) where fertilisation occurs externally. After fertilisation the zygote undergoes cellular division (Fig 1.3 B) with embryogenesis resulting in the formation of a free swimming planula larvae (Fig 1.3 C) typically taking 4 – 7 days (Babcock and Heyward, 1986; Okubo and Motokawa, 2007). Synchronous broadcast spawning events are commonly reported, during which a multispecific (multiple species and families of coral) annual release of gametes ensues over just a few nights of the year. This annual mass spawning was first described on the Great Barrier Reef in the early 1980s (Harrison *et al.*, 1984) and in the succeeding four decades it is now understood that multispecific synchronous spawning occurs in many locations around the world; Singapore (Guest et al. 2005), Palau (Kenyon, 1995; Penland *et al.*, 2004), Malaysia (Chelliah *et al.*, 2015), Philippines (Vicentuan *et al.*, 2008), Japan (Hayashibara *et al.*, 1993), the Caribbean (Bastidas *et al.*, 2005), the Persian Gulf (Bauman, Baird and Cavalcante, 2011) and the Red Sea (Bouwmeester *et al.*, 2011, 2014). The synchronicity of gamete release is an evolutionary adaptation of sessile marine invertebrates, aimed at ensuring cross fertilisation and minimising confounding effects of sperm dilution (Pennington, 1985; Serrão and Havenhand, 2009). In broadcast corals this coincides with rapid temperature rise (Keith *et al.*, 2016) (Fig 1.4 Aii) and is triggered by solar irradiance (van Woesik, Lacharmoise and Köksal, 2006) (Fig 1.4 Aiii), light spectrum dynamics of the photoperiod (Sweeney *et al.*, 2011) (Fig 1.4 Aiv) and the lunar cycle (Boch et al. 2011; Kaniewska et al. 2015) (Fig 1.4 Av). Furthermore, there appears strong ties with the tidal cycle, whereby spawning more commonly occurs on nights mapping on or near neap or smaller tides (Babcock *et al.*, 1986), again effectively minimising dilution effects. Finally, some studies have also drawn links to mass coral spawning during months prior to heavy rainfall, which possibly reduces the damaging effects of osmotic shock to the developing embryos (Mendes and Woodley, 2002).

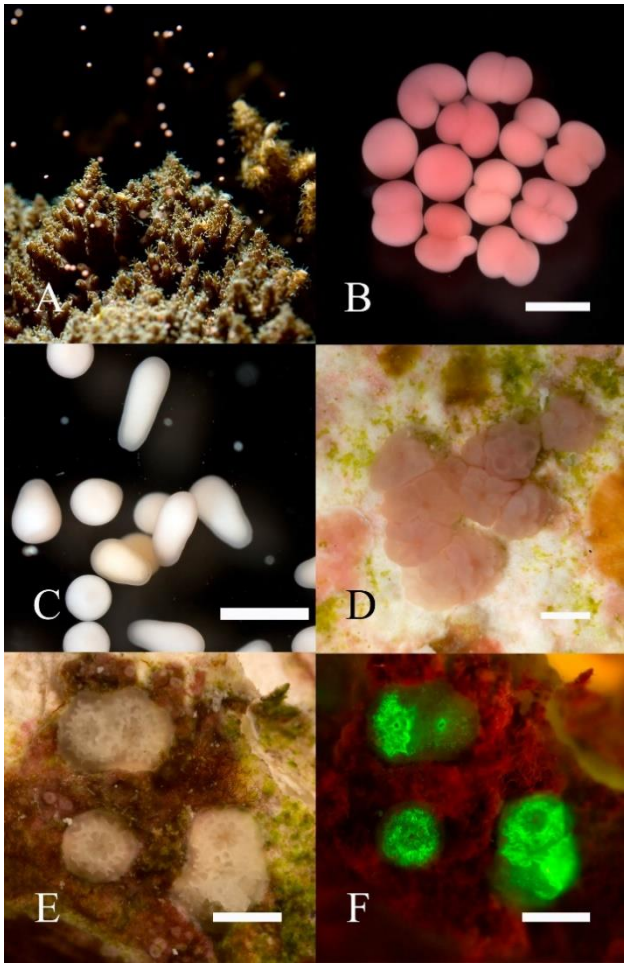


Figure 1. 3 Gamete release, planula and early post settlement development of the broadcast spawning coral, *Acropora hyacinthus*.

(A) *Ex situ* egg/sperm bundle release during synchronous annual spawning; (B) Recently fertilised zygotes, two and four cell blastomeres; (C) Newly developed planula larvae; (D) One day old primary polyps following metamorphosis; (E) Eight day old primary polyps under full spectrum lighting; (F) the same eight day old primary polyps imaged under Royal Blue light – excitation 440 – 460 nm, emission 500 nm longpass (NightSea, BlueStar Flashlight) highlighting Green Fluorescent Protein (GFP) at various stages of early development. Scale 1 mm. *All images taken by J Craggs*

During the pelagic phase, embryos and planula disperse in ocean currents, which marks an important process of reef connectivity and genetic flow between populations within geographical ranges. Measured gene flow, indicated as the number of migrations per generation (N_em), compared to distance, suggests that brooding species often recruit locally, settling within a few hours of release and therefore have limited dispersal potential (Nishikawa, Katoh and Sakai, 2003). By contrast the longer pelagic period (as a result of embryogenesis occurring externally in broadcast spawning species), show higher N_em, particularly over geological time scales and are important mechanisms in gene migration in these corals (Ayre and Hughes, 2000; Nishikawa and Sakai, 2005).

Chemical (Birrell *et al.*, 2008), and possibly acoustic (Vermeij *et al.*, 2010) cues stimulate larvae to drop out of the water column and metamorphose into a coral primary polyp (Fig 1.3 D). During this critical life stage the juvenile coral passes from the initial motile pelagic phase to a sessile benthic phase which predominates the life history of scleractinian corals. Planula of many broadcast species exhibit horizontal transmission of the Symbiodiniaceae, acquiring them from the water column post settlement (Littman, van Oppen and Willis, 2008), and therefore lack symbionts at this stage. In addition newly settled primary polyps, of species such as *Acropora spp.* do not contain GFP at settlement (like brooders), but rather these proteins develop during the first eight days (Fig 1.3 E & F). The functional role of GFP has been greatly debated but recent research indicates that Symbiodiniaceae actively swim towards light with the emission spectra of green fluorescence (Aihara *et al.*, 2019).

Following settlement primary polyps divide, through asexual budding (intra and extra-tentacular budding mentioned earlier), forming juvenile coral colonies that grow and become sexually mature from 3 years of age (Baria et al. 2012) (Fig 1.4Ai).

1.5 A rationale to develop *ex situ* broadcast spawning in corals

Due to increasing anthropogenic pressures facing coral reefs, coral scientists study annual mass broadcast spawning events to understand spatial and temporal trends, investigate ways of mitigating any damage and develop methods of repopulating reefs utilising sexual recruits for continued species survival (Heyward *et al.*, 2002; Omori and Iwao, 2009; Guest *et al.*, 2014; van Oppen *et al.*, 2015). A significant limitation to this area of study is that only small windows of time occur, during short annual synchronous spawning events, in which material is available for experimentation. Collecting gametes *in situ* also presents considerable logistical limitations associated with night diving, limited dive times underwater on standard scuba, and adverse ocean conditions. As a result many studies focusing on broadcast spawning species rely on the collection of gravid broodstock colonies a few days prior to gamete release, where they are transferred to flow-through aquariums in facilities close to natal reefs (Harrison *et al.*, 1984; Babcock and Heyward, 1986; Babcock *et al.*, 1986; Negri *et al.*, 2001; Okubo and Motokawa, 2007). This currently limits study in this area of reef ecology to scientists who have access to such facilities. This is in part due to the technical difficulty associated with managing closed system coral mesocosms for protracted experiments (D'Angelo and Wiedenmann, 2012; Rocha *et al.*, 2015). Some researchers have therefore concluded that such experiments with broadcast spawning corals is likely not possible without access to natural lunar light and the correct photoperiod (Leal *et al.*, 2014). The development of protocols that facilitate the *ex situ* replication of conditions associated with broadcast spawning events would in the first instance increase the number of institutions that are capable of studying reproduction. Understanding biotic and abiotic factors (that induce spawning events) and then being able to control these *ex situ* will increase the scope of scientific discovery associated with emerging areas of research such as assisted evolution and the growing importance of coral sexual reproduction for reef restoration efforts.

One reason why coral research (focused on reproductive processes) is usually confined to laboratories and facilities within close proximity to reef tract is down to the ability (or lack thereof) to transport parental colonies over large distances. Acquisition of broodstock for any captive reproduction programme relies on knowing that the individuals are reproductively mature and the transportation of those individuals to the facility in question. It has been shown that large coral colonies can be transported over long distances (Petersen *et al.*, 2004), however

to date no attempt has been made to ship gravid colonies between continents (Fig 1.4B). The acquisition, transportation and subsequent documentation of controlled broadcast spawning *ex situ* forms the initial basis of this thesis (chapter 2).

Following natural spawning events, methods are starting to emerge to allow the rearing of large numbers of planulae for research purposes or to supply material for reef restoration (Pollock *et al.*, 2017). While it is acknowledged that broadcast spawning events have been documented *ex situ* in public aquarium displays (Nosratpour, 2008), these are always unplanned and unpredictable events. Indeed, for reasons described earlier it has been suggested that inducing broadcast spawning in a fully closed mesocosm environment may not be possible (Leal et al, 2014).

However, understanding patterns of reproductive events for a given geographical location might provide valuable information (Fig 1.4 A & Ai), allowing for the creation of a framework to study coral reproduction *ex situ*. By defining a moment in time when spawning is predicted it might be possible to work backwards from that point, developing a logic pathway for a chosen synchronous location and to replicate the known biotic and abiotic influences that trigger spawning. Utilising the latest microprocessor technologies, a fully closed *ex situ* mesocosm that can replicate parameters such as seasonal temperature change (Fig 1.4 Aii), solar radiance curves (Fig 1.4 Aiii), photoperiod (Fig 1.4 Aiv) and lunar cycles (Fig 1.4 Av) would in theory enable planned inducement of broadcast spawning to occur, if coupled with appropriate husbandry approaches (chapter 3, Fig 1.4 C).

Spawning broadcast corals *ex situ* would mark an important milestone in the development of research potential in institutions away from natal reefs. However it is unknown whether spawning corals *ex situ* and the resulting embryos produced during *in vitro* fertilisation will develop naturally (Fig 1.4 D). Utilising scanning electron microscopy and confocal laser scanning microscopy, the embryogenesis of three acroporid species that were induced to spawn *ex situ* are evaluated in this thesis (chapter 4).

Increasing survivorship of settled larvae would be the logical next step to try and overcome for coral research both *ex situ* and closer to natal reefs. The nature of the complex interactions occurring post-settlement within aggregated polyp entities of multiple genotypes is one area of research that warrants further study (chapter 5), along with the advantages or disadvantages of

hybridisation (Fig 1.4 E).Following settlement, corals experience a highly competitive benthic environment with various algal species and predators having measurable negative impact on their survival (Wilson and Harrison, 2005; Vermeij *et al.*, 2009; Trapon *et al.*, 2013). Interestingly, some grazing species, which may cause damage to the spat, may also offer some level of assistance in survivorship by controlling the negative algae species, for example, sea urchins (Fig 1.4 F), which are in their own right important ecosystem engineering species (Valentine and Edgar, 2010). Could co-culturing be an answer to increasing survivorship of corals at this vulnerable stage? And is it a matter of scale? i.e. the importance of micro-herbivory (chapter 6).

Finally, it is the goal of this thesis to close the life cycle of a broadcast spawning coral. Within zoos and public aquariums many captive breeding programmes exist and these often focus on the conservation of endangered species (Tribe and Booth, 2003; McGregor Reid and Zippel, 2008; Conde *et al.*, 2011). An important process in these programmes is the management of genetic lines and closing the life-cycle is imperative for this to occur (Hora and Joyeux, 2009; Butts *et al.*, 2016). To date the production of gametes and multiple filial generations from *ex situ* reared broadcast reef building corals has not been documented (Fig 1.4 G). This milestone would mark an important progression in the sustainable management of *ex situ* populations by removing any dependency on *in situ* collection of corals either for public display or for research purposes (chapter 7). It may in turn lead to the development of land based coral nurseries that function as production facilities to produce coral seeding colonies for reef restoration purposes.

In this thesis, corals from two synchronous spawning locations, Singapore and the Great Barrier Reef, Australia were brought to the Horniman Museum and Gardens, UK and housed in purpose built mesocosms. Five broadcast spawning coral species were used as models in the chapters associated with this thesis and these include; *Acropora anthocercis* (Brook 1893) (Fig 1.5 A), *Acropora hyacinthus* (Dana 1846) (Fig 1.5 B), *Acropora microclados* (Ehrenberg 1834) (Fig 1.5 C), *Acropora millepora* (Ehrenberg 1834) (Fig 1.5 D) and *Acropora tenuis* (Dana 1846) (Fig 1.5 E).

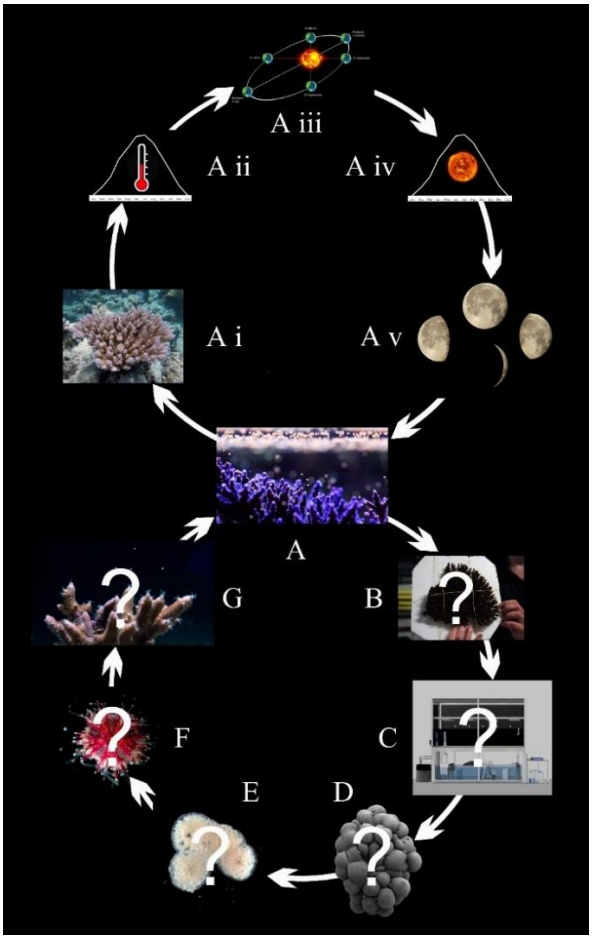


Figure 1. 4 Schematic of current known drivers of broadcast spawning *in situ* and questions that need to be answered for successful *ex situ* spawning of corals [unknown factors and questions *ex situ*].

(A) Synchronous broadcast coral spawning *in situ* occurs over a few nights each year; (Ai) species specific drivers, including colony age dictate maturity; (Aii) seasonal temperature changes trigger the onset of gamete development; (Aiii) gamete maturity and spawning season are driven by seasonal solar irradiance; (Aiv & v) photoperiod and lunar cycle defines the night and time of spawning; (B) is successful long distance transportation of gravid broodstock colonies possible?; (C) is *ex situ* spawning in closed systems possible without influence from natural photoperiod and lunar cycles?; (D) will normal embryological development occur in corals completing gametogenic cycles *ex situ*?; (E) what role does varying numbers of genotypes within settled entities and genetic crosses have on survival and genotype growth?; (F) can co-culturing utilising microherbivory enhance post settlement coral survival?; and (G) is it possible to close the life cycle of *Acropora ex situ*? *All images taken by J Craggs*

(A) *Acropora anthocercis,* colonies are corymbose (growth form with horizontal interlocking branches and short upright branches) to encrusting plates, with short thick branches, inhabits upper reef slopes exposed to stong wave action. Sometimes common in western Pacific, Indian Ocean and Red Sea; hermaphorditic broadcast spawner, annual spawning time on GBR has not been recorded.

(B) *Acropora hyacinthus,* colonies form wide flat plates or tables up to several meters in diameter, common on exposed outer reef slopes throughout the western Pacific; hermaphroditic broadcast spawner, annual spawning in Singapore March / April 4-6 nights after full moon (NAFM) at 21.00 hrs.

(C) *Acropora microclados,* corymbose forming colonies with short evenly spaced branches that are usually a distinctive pale pink – brown, inhabiting upper reef slopes in western Pacific; hermaphorditic broadcast spawner, annual spawning time on GBR has not been recorded.

(D) *Acropora millepora,* colonies are corymbose with short uniform branches, common species inhabiting shallow water reef flats, upper slopes and also lagoons in the western Pacific and Indian Ocean; hermaphorditic broadcast spawner, annual spawning time on outer GBR Nov / Dec 4-7 NAFM at 21.00 hrs.

(E) *Acropora tenuis,* colonies form corymbose clumps on upper reef slopes, common throughout western Pacific and Red Sea; hermaphorditic broadcast spawner, annual spawning time on outer GBR Nov / Dec 3-6 NAFM at 19.00 hrs.

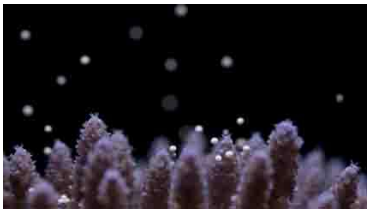


Figure 1. 5 Five species Indo-Pacific hermaphroditic broadcast spawning acroporids

(*Acropora anthocercis, Acropora hyacinthus, Acropora microclados, Acropora millepora* and *Acropora tenuis*) from two synchronous spawning locations, Singapore and Great Barrier Reef, Australia, were used for experimental investigations. Data from Veron (2000), Guest et al (2002), Babcock et al (1986). (A) *Acropora anthocercis* colony in Palau; (B) *Acropora hyacinthus* tagged colony AH11 on Pulau Satumu, Singapore; (C-E) show aquarium colonies, two in the process of spawning (D&E). *All images taken by J Craggs*

Chapter 2: Maintaining natural spawning timing in *Acropora* corals following long distance inter-continental transportation

This chapter has been published as: **Craggs J**, Guest JR, Brett A, Davis M, Sweet M, 2018 Maintaining natural spawning timing in Acropora corals following long distance inter-continental transportation. Journal of Zoo and Aquarium Research 6 (2) doi.org/10.19227/jzar.v6i2.317

2.1 Abstract

The majority of research focusing on coral reproductive biology (e.g. spawning timing and synchrony) is carried out in facilities adjacent to the reefs from which the corals originated. In part, this is because transporting corals over long distances by air leads to sub-lethal stress that may confound the results of any experimental study. However, these constraints often mean research associated with coral reproductive timing is restricted to relatively few locations.

To assess the potential for studying environmental drivers of spawning timing in corals in captivity (defined here as *ex situ* closed aquaria), I aimed to transport 14 large (16-37 cm) *Acropora hyacinthus* colonies from reefs in Singapore to a closed aquarium system in London (a journey time of ~34 hrs). Collection was purposefully timed to occur just before the predicted annual mass spawning event and on the day of transportation it was noted that 12 of the 14 corals contained large visible oocytes. The 'inverted submersion method' was applied and the water used for transport was buffered to ensure that the colonies remained healthy throughout their travel time. At the destination all colonies were placed into a purpose built aquarium research system which attempted to match the environmental conditions found on the fringing reefs south of Singapore (the original location). Whilst three colonies appeared partially bleached (visibly pale) and one colony suffered from partial tissue loss, all colonies (i.e. 100% of those collected) were still alive at the time of writing (28 months post collection). More importantly, all corals that were gravid at the time of collection spawned *ex situ* within the same lunar month as those in the wild (within 3 to 4 nights of each other). This chapter describes the procedures developed for carrying out long distance transportation of large gravid broadcast spawning coral colonies from reef sites to public aquariums or research facilities around the world for the purpose of *ex situ* spawning research.

2.2 Introduction

Research on scleractinian coral reproduction is a prerequisite for the study of life-history strategies associated with any given species, along with an understanding of the ecology and persistence of populations and communities, as well as the management and preservation of reefs (Rapuano *et al.*, 2017). Yet despite over three decades of research into the reproductive biology of broadcast spawning coral, conflicting views remain about the putative cues that drive these annual events (Penland *et al.*, 2004; van Woesik, 2010; Keith *et al.*, 2016). Conducting in-depth research in controlled *ex situ* environments (i.e. the use of mesocosms) is one way to disentangle the complex patterns seen in natural systems (Fordham, 2015). However, conducting research in this way when exploring reproduction of broadcast corals has presented numerous challenges. The first challenge is driven by the fact that many of the coral species in question have long gametogenic cycles (Wallace, 1985; Shikina *et al.*, 2012) and exhibit only single annual spawning events (Richmond and Hunter, 1990; Hayashibara *et al.*, 1993; Kenyon, 1995; Baird, Sadler and Pitt, 2001; Guest *et al.*, 2002, 2005; Penland *et al.*, 2004; Vicentuan *et al.*, 2008; Chelliah *et al.*, 2015). As a result, the majority of research in this field is conducted at locations close to the reefs of origin of these corals (Harrison *et al.*, 1984; Negri and Heyward, 2001; Okubo, Taniguchi and Motokawa, 2005).

The process of spawning has been shown to be affected by the physiological condition of the coral (Baird and Marshall, 2002), in addition to exogenous timing cues, which appear specific to any given location (Babcock *et al.*, 1986; Kaniewska *et al.*, 2015). However, little is known about how handling and transportation of the corals for use in *ex situ* experiments affects the synchrony and spawning success. If it could be demonstrated that corals could be transported over both long and short distances successfully, without having any detrimental effect on the reproductive cycle, this field of coral biology could expand greatly as it would enable experiments to be conducted in a wider range of locations and repeated experiments on the same colonies. With these aims, researchers can turn to coral aquaculturists, zoos, aquariums and the hobbyist trade for guidance. However, even in these fields, there appears to be reliance on bulk transportation strategies rather than ensuring a high level of fitness at the end point. Indeed in certain instances issues associated with bacterial loading during transportation have resulted in massive disease outbreaks (Delbeek, 2008). Traditionally, two packing techniques are utilised when transporting corals over long distances – the ‘wet packing method’ and the

'dry packing method' (Delbeek and Sprung, 1994; Carlson, 1999; Petersen *et al.*, 2004; Delbeek, 2008). The majority of shipments employ the wet method and in many cases, corals shipped from coral farms and public aquariums are mounted onto bases which are suspended upside down in the transportation bag attached to a Styrofoam® float (Delbeek, 2008). Referred to as 'inverted submersion' (Calfo, 2001), the Styrofoam® raft is larger than the suspended coral preventing contact with the sides of the transportation bag and preventing damage during transit. In this study, I assess the potential of the inverted submersion method for transporting gravid colonies of *Acropora hyacinthus* over long distances (~34 hrs travel time). Success of the transportation is measured by comparing the health state and the synchrony of the spawning events in the *ex situ* mesocosm and the parental colonies which remained on the reef.

2.3 Material and methods

2.3.1 Species and study site

Acropora hyacinthus is a common reef building species that is found on reef slopes or reef flats throughout the Indo Pacific. It forms flat wide plates (over 3m in diameter), tables or tiered aggregations of smaller plates (Veron, 2000). For this study, all colonies were collected from two reefs south of mainland Singapore (Kusu reef and Pulau Satumu). In order to collect and ship live corals, the appropriate research permit was sought from the National Biodiversity Centre, National Parks Board Singapore (NP/RP14-115) and a CITES import (permit number: 532422/01) and export permit (permit number: 15SG006834AE) were also obtained for the shipment.

2.3.2 Stage 1: Local transportation and preparation for international transport

The collection of *A. hyacinthus* for this study was timed to be within one month of the actual spawning times in the wild (Guest *et al.*, 2002, 2005), with the intent that some of the corals would be gravid during transport and that the corals would be in the late stage of oocyte development (Wallace, 1985), reducing the chance of oocytes being reabsorbed.

On 27th February 2015, ten *A. hyacinthus* colony fragments were removed from parental colonies at Kusu reef using a hammer and chisel. To minimise the impact to the existing coral populations, no more than 10% of the total surface area was taken from any one colony, as this has been shown to have minimal effects on the health status of the parental colonies (Epstein, Bak and Rinkevich, 2001). Parental colonies were primarily selected based on the indications of good overall health (i.e. no tissue recession or bleaching). Furthermore, each colony sampled was separated by more than 8m (horizontally along the reef) in order to minimise the chance of sampling genetically similar colonies (Ayre and Hughes, 2000). The collected fragments were measured and photographed and the parental colonies were tagged (*AH1-10*). The fragments were moved to a temporary nursery located at approximately the same depth as the parental colonies (~3m). The nursery was constructed with an aluminium angle bar and colony fragments were attached using cable ties. Fragments were left in the nursery for 18 days prior to transportation (Fig 2.1). This was in order to allow damage caused during fragmentation to heal.

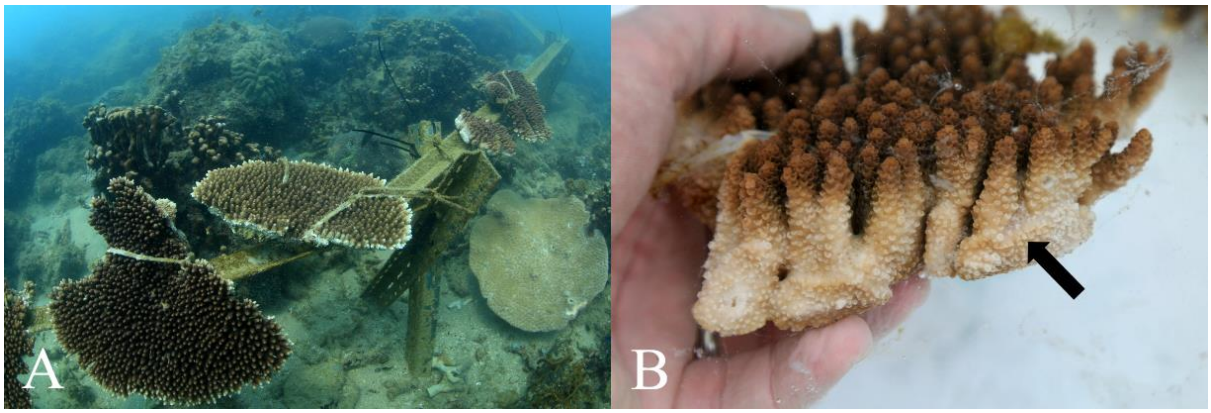


Figure 2. 1 *Acropora hyacinthus* colony fragments in an *in situ* nursery following collection.

(A) *A. hyacinthus* colony fragments attached with cable ties to temporary nursery; (B) illustrating the new epithelial tissue (black arrow) which grew over all areas of exposed skeleton in the 18 days from initial removal from parental colony to date of transportation. *All images taken by J Craggs.*

One day prior to transportation, the water and packing materials were assembled. A reservoir for 'de-sliming' and a reservoir for packing water was prepared with 5 µm filtered seawater. The packing water was sterilised with a UV steriliser for 24 hrs to reduce bacterial levels.

Sodium bicarbonate was added to raise the alkalinity to 9 dKH (3.214 meq/l or 160.714 ppm $CaCO_3$) to minimise changes in pH during transport. On the day of the shipment (17th March 2015), four additional colony fragments were removed from a further four parental colonies at Raffles Lighthouse Reef (Pulau Satumu) (AH11-14) using the method described above, and the ten original colony fragments were collected from the temporary nursery site at Kusu reef. Any visible microfauna were removed from the coral colonies to prevent fouling of transport water. All colonies were transported back to the regional public aquarium (the S.E.A. Aquarium – Sentosa Island, Singapore) in insulated boxes fully submerged in seawater and covered with a lid. During this stage of the transportation, all fragments produced substantial amounts of mucus. Mucus was therefore removed from the containers and additional seawater added before the next stage of transportation. Temperature and pH were monitored during this period (using YSI Pro1030) and multiple water changes were conducted based on the results and the quantity of mucus being produced. Ice placed in plastic ziplock bags was also floated inside the boxes in order to maintain a constant temperature during transit. The total time for transportation from collection to arrival at the local aquarium was one hour for AH 1 - 10 and 3 hrs for AH 11 - 14. Upon arrival the transportation water was slowly exchanged with filtered seawater to acclimate the colonies.

2.3.3 Stage 2: International long distance transportation and acclimation procedure

Each colony fragment was attached, upside down, to a 25mm thick Styrofoam® flotation raft with three or four large thin rubber bands (Fig 2.2). Flotation rafts were made to be larger (>5cm dia) than the coral to prevent the coral from touching the sides of the box, as contact with the box can cause damage resulting in secondary bacterial infections (Delbeek, 2008). Attached corals were then suspended upside down in the ‘de-sliming’ reservoir for 20 to 30 mins. Hanging the corals in the reservoir allows them to release the mucus produced as a result of handling, and thereby ultimately reducing the amount of mucus released during the next stage of transportation (Delbeek, 2008).

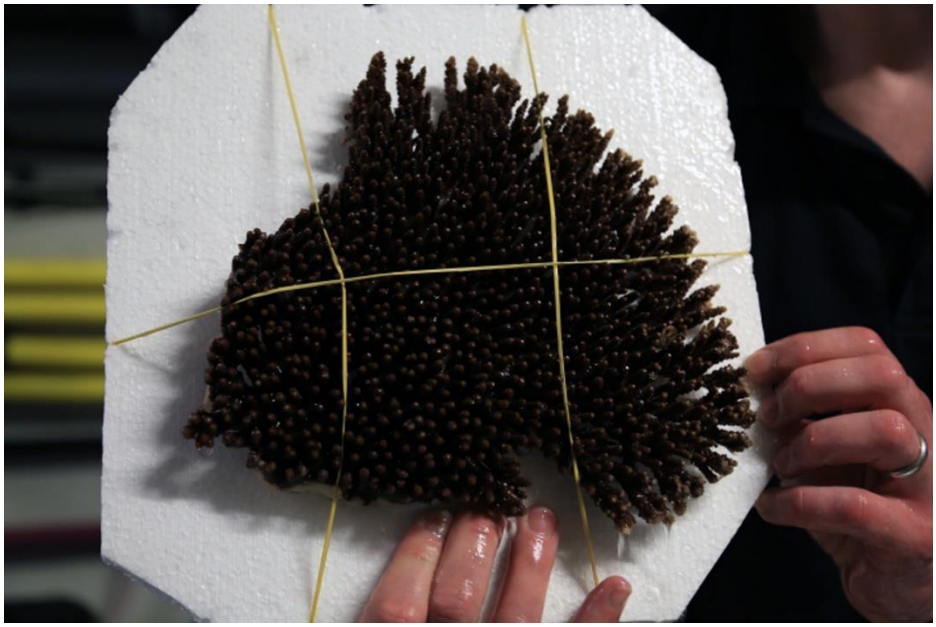


Figure 2. 2 Large colony fragments are attached to Styrofoam® floatation raft with elastic bands and floated upside down in the transportation bag.
Image taken by J Craggs

Styrofoam packing boxes were prepared with 15 litres of buffered, sterilised seawater in a 61cm (B) x 107cm (L) plastic bag (note corals are always double bagged in case of leakage). The initial parameters of the water in these bags were as follows: temperature 27.5 °C; dissolved oxygen 96%; pH 7.97; and alkalinity 9 dKH (3.214meq/l). Individual corals were packed separately and placed upside down in the bag – the presence of the Styrofoam® ensuring that the corals floated upside down. Pure oxygen (100%) was added before sealing in an oxygen to water ratio of between 1:2 and 1:3 (parts water : oxygen). Two heat packs were also taped to the lid of the box to maintain temperature during the flight to the United Kingdom (UK). Boxes were sealed and labelled with the appropriate parental colony tag number and sent for transport with a freight forwarder.

Upon arrival in the UK (~34 hrs of travel time), the corals were immediately unpacked and the water parameters, temperature, pH (Hach Lange HQ11d), and dissolved oxygen (OxyGuard, Handy Gamma) were measured (Table 2.1). Colony AH11 was transferred directly to a coral research system without acclimatisation. This was due to the amount of zooxanthellae released during transit and the subsequent dark brown water (Fig 2.3). The remaining corals (AH1-10, and 12-14) were acclimated to aquarium water for 2 hrs, using 6 mm silicone tubing with

system water being added at approximately 80 ml/min (0.5% of transportation water) via a gravity siphon. Acclimation was determined to be complete once transportation water closely matched system parameters (28.7 °C, NH_3 0mg/l, NO_2^-0mg/l, NO_3^- 0.02mg/l, PO_4^{3-} 0.035mg/l, 32ppt salinity, pH 8.1 and alkalinity 7dkh). Colonies were then attached to pieces of live rock using cable ties and transferred to the 1200 litre coral research system. Internal water movement was provided by four internal flow pumps (Jebao RW-20), each rated to 20,000 litre/hr. The coral research system was specifically designed for broadcast coral spawning research and has the ability to accurately replicate environmental parameters associated with broadcast coral spawning, including seasonal temperature change, photoperiod and lunar cycle and annual solar irradiation changes matching those of Kusu reef (Craggs et al. 2017, Chapter 3).

Table 2. 1 Water chemistry post-transport from the S.E.A. Aquarium (Singapore) to the Horniman Museum and Gardens (London, United Kingdom).

Coral ID	Temperature (Celsius)	Salinity (ppt)	pH	Dissolved Oxygen (%)
AH1	24.6	32.2	6.94	304
AH2	25.6	32.2	7.03	293
AH3	24.5	32.2	7.08	283
AH4	27.0	32.2	7.02	313
AH5	26.1	32.2	7.07	208
AH6	26.2	32.1	6.93	258
AH7	24.8	32.2	6.93	260
AH8	26.7	32.2	6.95	214
AH9	26.0	32.1	7.40	336
AH10	25.7	32.1	7.05	250
AH11	-	-	-	-
AH12	26.3	32.2	7.16	288
AH13	28.2	31.3	7.16	123
AH14	25.7	32.1	6.97	271

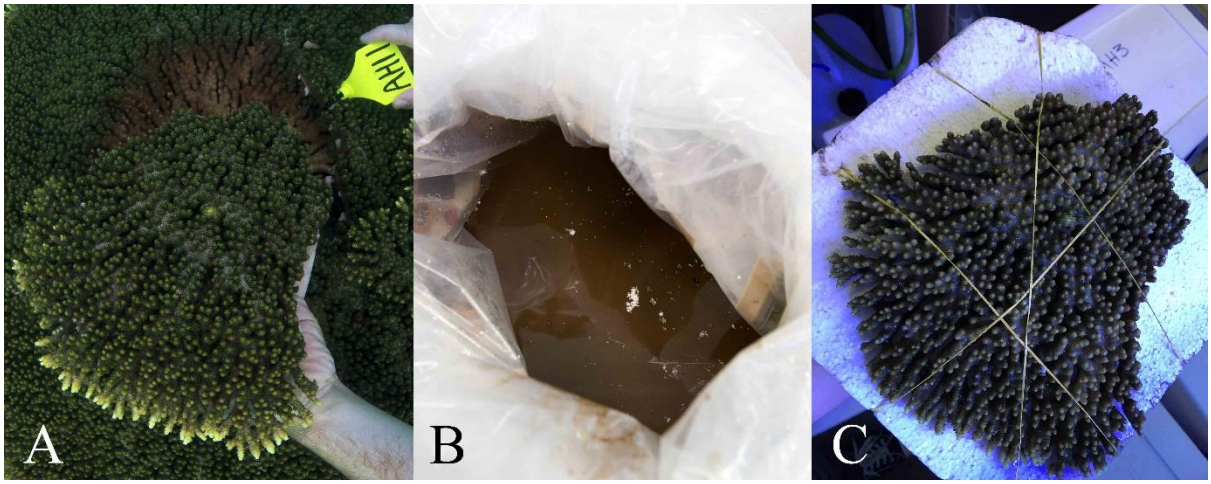


Figure 2. 3 *Acropora hyacinthus* colony AH11 collected in Singapore and following transportation of United Kingdom.

(A) Colony fragment AH11 recently removed from the parental colony. Parental colony tag seen in top right; (B) Packing water at the end of the 34 hr shipment showing high levels of zooxanthellae expulsion; (C) Colony on arrival in the UK with pigmentation loss during transportation. *All images taken by J Craggs*

Two days after arrival daily heterotrophic feeds were presented consisting of yeast culture (16 ml/1,000 litres), AcroPower by Two Little Fishes amino acid solution (16 ml/1,000 litres), live phytoplankton *Tetraselmis spp* (200 ml/1,000 litres), live *Artemia salina* nauplii (300 nauplii per litre) and frozen *Brachionus plicatilis* (1,000–2,000 per litre). During feeding the holding tank was isolated from the filtration for approximately two hrs to aid feeding uptake.

2.3.4 Histological sampling to assess egg development

Completion of Acropora gametogenic cycles take up to five months, during which oocyte diameter increases (Wallace, 1985) and changes in oocyte colouration occur, initialling starting as white and progressing to either orange, pink or red pigmentation in the month prior to spawning (Harrison *et al.*, 1984). To assess the stage of oocyte maturity three individual coral fragments (<3 cm in length) were taken from randomly selected areas of each colony on three occasions (25th March, 4th April & 23rd April 2015). Care was taken to avoid sterile zones on the periphery of the colonies (Wallace, 1985). To check for the presence of oocytes, cross sections of each sample were photographed using a Canon 5d MKIII with MPE 65mm lens set

to x5 magnification. Cross sections were illuminated using a Schott KL1500 LCD cold light source. Kelvin temperature of both light source and camera were calibrated to give true subject colour rendition. After imaging, the samples were then preserved in 10% formalin and histological sections were prepared and stained with haematoxylin and eosin by International Zoo Veterinary Group following methods described by Chornesky and Peters (1987).

2.4 Results

2.4.1 Preparation for transport

All corals were prepared as planned, except one. AH7, was too large to be floated in the bag as the coral touched the bottom when upside down. This colony was packed upright, fully submerged.

2.4.2 Transportation and acclimation procedure

The coral shipment arrived after a total transport time of 34 hrs from the initial time of collection (reef) to when they were unpacked at the final destination (the Horniman Museum and Gardens, UK). The pH, temperature and dissolved oxygen varied between transportation boxes (Table 2.1). Temperature ranged from 24.8 to 28.2, pH 6.93 to 7.40, and dissolved oxygen 123% – 336%. All colonies released their algal symbionts (or Symbiodiniaceae) to some degree, but there was variation from colony to colony. The most severe case was regarding AH11, whereby the packing water was visibly dark brown in colouration (Fig 2.3). As such AH11 was immediately removed and placed in the holding tank due to concerns for colony health. One bag was punctured during transit (containing AH13), but the colony did not appear to show any physical damage.

2.4.3 Post transportation survival rates and colony health

Pigmentation was deemed to have returned to ‘normal’ after approximately two months based on comparisons using photographs taken of parent colonies at the time of collection and the fragmented colonies in the tanks.

One day after arrival in the UK colonies started to exhibit the first signs of polyp extension, with full extension occurring within a further two weeks across all colonies. Colony AH10 suffered a disease outbreak, similar in appearance to white syndrome (Sweet *et al.*, 2013) 13 days after transport. The infected area was cut away using a hacksaw, clearing the infected tissue by ~3 cm (Fig 2.4) and the infection was immediately halted with full healing occurring 3-4 weeks after the event.

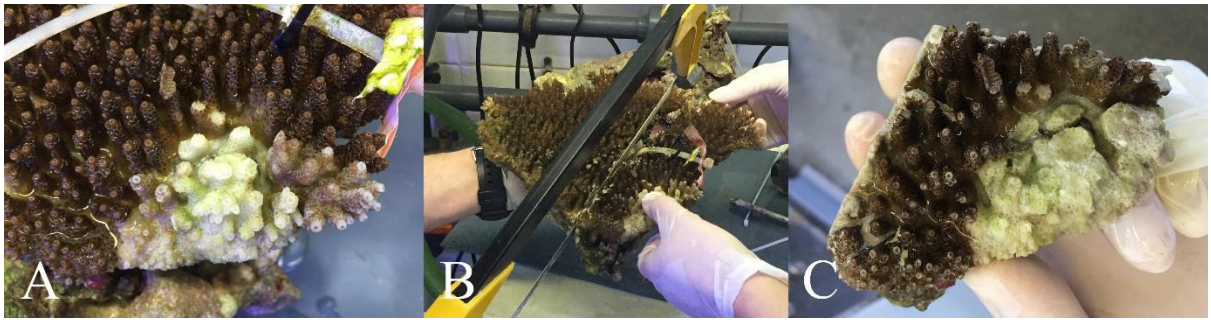


Figure 2. 4 Infected *Acropora hyacinthus* colony AH10 following transportation

(A) White syndrome on AH10 13 days post transportation; (B) Infected area being removed with a hacksaw; (C) Removed infected fragment including healthy band of tissue around the infection. *All images taken by J Craggs*

A total of 13 hairy coral crab**,** *Cymo andreossyi* (Audouin 1826) were removed from the corals. Despite this species having previously been described as an obligate commensal of branching corals (Hogarth, 1994) these crabs were seen feeding on the coral tissue, causing damage which manifested as denuded areas of skeleton. Despite partial mortality due to disease and predation (by the hairy coral crab, *C. andreossyi*), all colonies were alive at the time of writing of this Chapter (28 months post transportation).

2.4.4 Histological sampling to assess egg development

Upon arrival on 25th March 2015 coral samples showed numerous orange or pink pigmented oocytes (Fig 2.5) in 12 out of the 14 colonies (Table 2.2 & Appendix 1) which were in the late stages of oogenesis at time of transportation (Wallace 1985). Sampling on the 4th April 2015 confirmed this but in addition oocytes where also detected in AH7 (Table 2.2 & Appendix 2). Colony AH9 spawned *ex situ* at 21.10 on 10th April 2015, 6 nights after full moon (NAFM)

and colonies AH1,2,4-8,10-14 spawned on 12th-13th April 2015, 8-9 NAFM. These latter instances of spawning were indirectly observed as evidenced by the turbid water within the research aquaria the morning after egg/sperm bundle release. This was later confirmed by checking the cross sections of the above colonies ten days after spawning as there were no eggs seen in these corals to indicate that the corals had spawned (Appendix 3).

Table 2. 2 *Acropora hyacinthus* gamete sampling.

Colonies AH1 – 14 sampled on 25th March, 4th April and 23rd April 2015 for the presence or absence of oocytes prep and post *ex situ* spawning. mature pigmented oocytes (oo), oocytes not present (x)

Colony code	25th March 2015	4th April 2015	23rd April 2015
AH1	oo	oo	x
AH2	oo	oo	x
AH3	x	x	x
AH4	oo	oo	x
AH5	oo	oo	x
AH6	oo	oo	x
AH7	x	oo	x
AH8	oo	oo	x
AH9	oo	oo	x
AH10	oo	oo	x
AH11	oo	oo	x
AH12	oo	oo	x
AH13	oo	oo	x
AH14	oo	oo	x

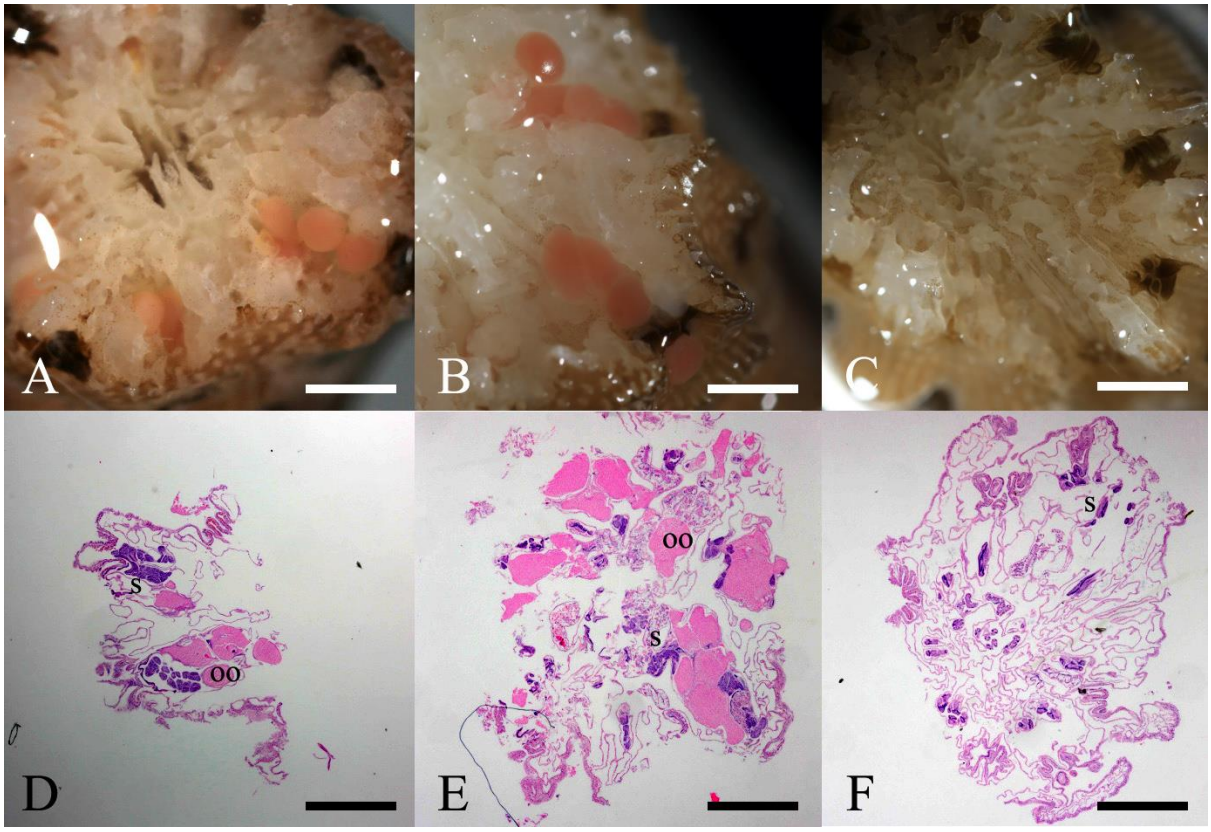


Figure 2. 5 Tracking gamete development within post transported *Acropora hyacinthus* colony fragments.

Three fragments were removed from each colony (AH1-14) on the three separate dates, 25th March, oocytes in late stage of development (A & D), 4th April oocytes in late stage of development (B & E) & 23rd April 2015 oocytes absent (C & F). Here colony AH9 is represented. oo = oocytes, s = spermaries. Scale 1mm. *All images taken by J Craggs*

In situ observations from Raffles Lighthouse Reef 3-6 NAFM (7th-10th April 2015) confirmed that 20 species of scleractinian coral spawned 4 NAFM and *Acropora* species, including *A hyacinthus*, 6 NAFM (K. Tun pers. comm.).

2.5 Discussion

In this study the transportation of large gravid colonies of *Acropora hyacinthus* during a period of ~34 hrs travel time from Singapore to the UK was successful. All colonies are alive at the time of writing with only minor signs of compromised health recorded during the entire

process. The conditioning of corals prior to shipment, involving the removal of excess mucus, has been suggested as being critical in order to reduce the bacterial load during transport (Delbeek, 2008). *A. hyacinthus*, as with all corals, produce a large amount of mucus when stressed (Brett pers obs). When being transported, this mucus remains closely associated with the coral surface most likely causing further stress and possibly exposing the corals to potential pathogenic organisms. Previous studies have not only documented dramatic shifts in the corals' surface mucus (CSM) microbiome over short and long time periods (Williams et al. 2015) Sweet et al. 2017) they have also shown that the composition of the mucus can change when corals are stressed (Lee *et al.*, 2016). Other studies have drawn the link with opportunistic coral pathogens associated with the CSM and together with the stressed state this may explain the onset of disease in one of the colonies post transportation (Banin *et al.*, 2001; Rosenberg *et al.*, 2007; Glasl, Herndl and Frade, 2016). However few studies have explored shifts in the microbiome in regard to transportation of corals and this warrants further study to see if this part of the process could be improved in the future. For example the use of beneficial coral microbes (BCM) has recently been suggested with regard to reef health (Peixoto *et al.*, 2017), although the risks of such procedures in nature have been highlighted (Sweet et al. 2017). Such inoculation during transportation would be an interesting aspect to explore. The study by Delbeek (2008) also suggested the idea of utilising a more extreme conditioning procedure before long distance transportation is undertaken, whereby the corals are intentionally stressed in order to release their mucus reserves. This was not tested in this study as my aim consisted of successfully transporting gravid corals to the UK. However, a smaller more targeted study exploring these options would be warranted in order to improve the technique described in this study.

Interestingly, in this study 10 colony fragments were removed from parental colonies and placed in a nursery for a period of 18 days prior to shipping to allow the fragment time to heal, whilst four additional fragments were collected on the day of transportation. Despite the differences in collection time prior to transportation all colonies survived and therefore the necessity of such additional precaution must be questioned.

A common cause of stress in scleractinian corals is physical damage (Chabanet *et al.*, 2005). Here, I attempted to minimise physical damage caused during transit by inverting the corals and attaching them to polystyrene floats. This allowed for the transportation of much larger

colonies than previously documented, although Petersen et al (2004) suggested that this technique was limited to corals under 1kg. However, the method does come with some slight costs, the direct increase in freight costs and the indirect increase in the risk of damage through careless handling during transit (Delbeek, 2008).

The overall intention of this study was to not only illustrate that large colonies could be transported significant distances with minimal effects on the health state but also to start a breeding stock of corals for an aquarium collection and for further reproduction studies associated with the new *ex situ* collection. In order to attempt this, parental colonies which were gravid at the time of transportation were chosen. Not only did this allow me another way to monitor health during transportation i.e. the lack of reabsorption of the eggs as witnessed in other studies (Okubo, Taniguchi and Motokawa, 2005) but I could (with careful husbandry) also induce spawning to synchronise with the spawning of the parental colonies in the wild. This was achieved within a few days (2-3 nights after) of the natural spawning event which was monitored at the original site. The ability to spawn corals *ex situ* can allow researchers the unique opportunity to explore reproductive biology anywhere in the world, providing care is taken with transportation and the husbandry of the corals at the host site. This advances the previous capabilities of research in this area which was limited to the transportation of coral larvae over similar distances which had recorded success of >90% survivorship at densities of 4 larvae ml^{-1} (Petersen *et al.*, 2005).

In conclusion, large gravid *A. hyacinthus* colonies can successfully be transported long distances (up to 34 hrs in this instance), with no mortality and will spawn within the same lunar months as their natal reef. Such an ability offers a suite of opportunities to coral reef scientists; from gaining a greater understanding of the ecology and persistence of certain coral populations and communities, to supporting novel methods for the management and preservation of reefs on a global scale.

Chapter 3: Inducing broadcast coral spawning *ex situ*: a closed system mesocosm design and husbandry protocol

This chapter has been published as: **Craggs J**, Guest JR, Davis M, Simmons J, Dashti E, Sweet M. 2017. Inducing broadcast coral spawning *ex situ*: Closed system mesocosm design and husbandry protocol. Ecology and Evolution 1–13. http://doi.wiley.com/10.1002/ece3.3538

3.1 Abstract

For many corals, the timing of broadcast spawning correlates strongly with a number of environmental signals (seasonal temperature, lunar and diel cycles). Robust experimental studies examining the role of these putative cues in triggering spawning have been lacking until recently because it has not been possible to predictably induce spawning in fully closed artificial mesocosms. Here, I present a closed system mesocosm aquarium design that utilises microprocessor technology to accurately replicate environmental conditions, including photoperiod, seasonal solar irradiance, lunar cycles and temperature regimes from Singapore and the Great Barrier Reef, Australia. Coupled with appropriate coral husbandry, these mesocosms were successful in inducing, for the first time, broadcast coral spawning in a fully closed artificial *ex situ* environment.

Four *Acropora* species (*Acropora hyacinthus, Acropora tenuis, Acropora millepora, Acropora microclados*) from two geographical locations, kept for over one year, completed full gametogenic cycles *ex situ*. The percentage of colonies developing oocytes varied from approximately 29% for *A. hyacinthus* to 100% for *A. millepora* and *A. microclados*. Within the Singapore mesocosm, *A. hyacinthus* exhibited the closest synchronisation to wild spawning, with all four gravid colonies releasing gametes in the same lunar month as their wild counterparts. Spawning within the GBR mesocosm commenced at the predicted wild spawn date but extended over a period of three months. Gamete release in relation to the time post sunset for *A. hyacinthus, A. millepo*ra and *A. tenuis* were consistent with time windows previously observed in the wild. However, spawn date in relation to full moon was delayed in all species, possibly as a result of external light pollution.

The system described here could broaden the number of institutions on a global scale that can access material for broadcast coral spawning research, providing opportunities for institutions

distant from coral reefs to produce large numbers of coral larvae and juveniles for research purposes and reef restoration efforts.

3.2 Introduction

Sexual coral reproduction, dispersal and successful recruitment is a fundamental process on coral reefs that ensures the long-term maintenance of biodiversity (Hughes *et al.*, 2000). The majority of scleractinian corals broadcast spawn gametes during short synchronous annual events (Harrison *et al.*, 1984; Babcock *et al.*, 1986; Guest *et al.*, 2002; Chelliah *et al.*, 2015), following a gametogenic cycle of up to nine months (Wallace, 1985). Synchronising spawning within a short temporal window is likely to be a highly adaptive strategy for the corals, yet environmental mechanisms that drive this behaviour are still not fully understood. It is generally accepted that seasonal, lunar and daily environmental rhythms work over progressively finer scales to determine the development of gametes, the night and the exact time of spawning (Harrison *et al.*, 1984; Babcock *et al.*, 1986; Oliver *et al.*, 1988).

Several factors have been proposed to drive the seasonal timing of gametogenesis including; solar irradiance (Penland *et al.*, 2004), sea surface temperatures (SST) (Harrison *et al.*, 1984; Keith *et al.*, 2016), regional wind fields (van Woesik, 2010), tidal rhythms and seasonal patterns in rainfall (Mendes and Woodley, 2002). Environmental rhythms related to the lunar cycles are undoubtedly involved in determining the date of the spawning (Babcock *et al.*, 1986) and diel light cycles have been shown experimentally to drive the actual timing of such spawning events (Boch *et al.*, 2011). Studies suggest that the timing of spawning may be driven by a light mediated biological process which reacts to the differential shift of darkness post twilight and premoonrise (Boch *et al.*, 2011; Kaniewska *et al.*, 2015; Brady *et al.*, 2016), and at a secondary level to changes in spectral dynamics of twilight and lunar phases (Boch *et al.*, 2011; Sweeney *et al.*, 2011).

Controlled mesocosm experiments are necessary in order to assess the specific role of proximal cues on spawning timing and synchrony. However, the majority of studies to date have relied on correlations, despite the fact that many seasonal factors are collinear and therefore difficult to disentangle. For example, both van Woesik (2006) and Penland (2004) showed correlations between the times of peak solar irradiance and spawning events in the Caribbean and Palau

respectively. In contrast, Keith et al (2016) found that for Indo-Pacific *Acropora* assemblages, peak month of spawning coincided with the largest month-to-month increase in SST. Intermediate wind speeds also contributed to the prediction of spawning months, although the relationship was weak (Keith *et al.*, 2016). Despite uncertainty about the precise role of proximal drivers it is often possible to predict, with a high level of accuracy (i.e. within minutes from year to year), the exact time particular species on particular reefs will spawn (Vize *et al.*, 2005).

However, the majority of the studies are either conducted *in situ* or during short term *ex situ* experiments. The dearth of manipulative experimental studies largely stems from the technical challenges associated with maintaining corals *ex situ* (in a healthy state) in mesocosms over extended time periods (D'Angelo and Wiedenmann, 2012). Indeed a mesocosm that can support a thriving coral population, requires careful methodological design, incorporating appropriate filtration equipment to control the water chemistry parameter and meet the biological requirements of coral species being studied (Rocha et al 2015). Additionally, only a few *ex situ* experiments have been successfully focused on coral reproduction and these have primarily focused on a limited number of brooding coral species (Petersen *et al.*, 2006). Replicating the environmental parameters that have been associated with broadcast spawning in an *ex situ* mesocosm generates another level of technical challenges. Indeed some researchers have even noted that such closed *ex situ* systems, particularly for broadcast spawning corals, may not be possible without access to natural lunar light and the correct photoperiod (Leal *et al.*, 2014). As such to date there has been no recorded case of a broadcast spawning coral completing full gametogenic cycle and purposefully been induce to spawn in an *ex situ* closed environment.

Here I present a novel design for a mesocosm aquarium that can replicate *ex situ* environmental parameters thought to drive spawning synchrony (seasonal SST, photoperiod, lunar cycle and solar irradiance). This is in order to facilitate controlled spawning events in four species of broadcast spawning corals from two geographically distinct locations; Singapore and the Great Barrier Reef (GBR). This system allowed me (with a strict tailored husbandry protocol), to successfully spawn all four acroporid species (*Acropora hyacinthus, A. millepora, A. microclados* and *A. tenuis*) in a fully closed artificial *ex situ* environment.

3.3 Materials and methods

3.3.1 Study sites and coral species

The annual mass spawning in Singapore occurs 3 to 5 NAFM in late March, early April (Guest *et al.,* 2002). Whilst the annual mass spawning on the inner GBR occurs 4 to 6 NAFM in late October / early November (Harrison *et al.,* 1984; Babcock *et al.,* 1986). From these locations I chose four common reef building *Acropora* species as broodstock. These included: *Acropora hyacinthus, A. millepora, A. tenuis* and *A. microclados*. Fourteen *A. hyacinthus* colony fragments (AH1-14) were sourced from Kusu (latitude 1.223874, longitude 103.862622) and Pulau Satumu (latitude 1.160469, longitude 103.740416) Singapore (CITES import permit number: 532422/01). Five colony fragments of *A. millepora* (AM1-5), seven *A. tenuis* (AT1-7) and six *A. microclados* (AMIC1-6) from the reefs close to Cairns, GBR Australia (CITES import permit number: 537547/02 & 537533/02). Colony fragments, ranging in diameter from 10 to 39 cm were removed from parental colonies using a hammer and chisel. Following a recovery period of five to 14 days in a nursery, colony fragments were shipped using the inverted submersion technique (Calfo, 2001) (see Chapter 2). Collection and shipping was timed to take place 1 to 2 months before the predicted wild spawning date for each location (Harrison *et al.*, 1984; Babcock *et al.*, 1986; Guest *et al.*, 2002, 2005). The purpose of shipping corals prior to known spawning dates was to ensure they spawned at the start of the study and were therefore able to undergo a full annual gametogenic cycle *ex situ*. This approach ensured that individual colonies were sexually mature and would reproduce during known spawning periods. The system's ability to replicate the environmental conditions associated with the development and release of gametes *ex situ* was then determined based on three factors: 1) individual colonies completing full gametogenic cycle *ex situ*, 2) successful spawning *ex situ* in a high proportion of colonies and 3) the timing of spawning *ex situ* matching that on natal reefs.

Figure 3. 1 Schematic of mesocosm design.

(A) 780 litre broodstock aquarium; (B) main drive pump; (C) 40mm polyvinyl chloride (PVC) stand pipes; (D) mechanical filtration section of sump; (E) algae refugium; (F) protein skimming section of sump; (G) main drive pump section of sump; (H) E200 PowerRoll filter; (I) Wave P2oint luminar; (J) protein skimmer; (K) baffle; (L) 32mm polyvinyl chloride (PVC) inlet; (M) fluidised reactor; (N) Triton Base elements CORE 7; (O) four channel peristaltic pump; (P) multi chamber container for individual element corrective dosing; (Q) Internal wave maker pump, Jebao WR20; (R) aquarium chiller; (S) Radion XR30w Pro LED light; (T) lunar LED; (U) integrated black mdf panel fitted into an aluminium frame; (V) blackout blind.

3.3.2 Mesocosm design

Two mesocosm aquariums were built at the Horniman Museum and Gardens, London, one for each study location. The 780 litre broodstock aquariums (240cm L × 65cm W × 50cm D) (Fig 3.1 A) were supplied via a main drive pump (EcoTech Marine Vectra L1) (Fig 3.1 B) giving a flow rate of 16,000 litre hr^{-1} with the sump below. Two 40mm diameter stand pipes (Fig 3.1

C) allowed water to return from the broodstock aquarium into the sump (222cm L × 62cm W × 43cm D). The sump contained the filtration for the mesocosm aquarium and was divided into four sections for mechanical filtration (Fig 3.1 D); algae refugium (Fig 3.1 E), protein skimming (Fig 3.1 F) and the main drive pump (Fig 3.1 G). Water returning from the broodstock aquarium entered the first section of the sump, housing a particulate filter (D&D The Aquarium Solution, E200 PowerRoll Filter) (Fig 3.1 H), the purpose of which was to remove particulates (uneaten food, detritus and fish faeces) before they could break down to form nitrate (NO_3^-) and phosphate (PO_4^{3-}). Water then flowed into an algae refugium housing a mix of macroalgae (*Caulerpa prolifera*, *C. brachypus*, *C. racemosa* and *Chaetomorpha spp*) that were lit by four 54 watt T5HO fluorescent bulbs (Wave Point 54 watt Luminar, x2 Sun Wave & x2 Super Blue) (Fig 3.1 I) on a 12/12 hr light/dark cycle. As algae grew NO3 and PO4 were taken up from the water and exported from the mesocosm via regular algae harvesting.

Water then flowed into the third section, via a meshed weir, that housed a protein skimmer (Fig 3.1 J) (ATB Normal Size) specified to the capacity of the mesocosm. A baffle (Fig 3.1 K) at the opposing end of the weir increased skimming efficacy by trapping surface tension, allowing organic compounds to accumulate at the surface due to the hydrophilic and hydrophobic poles of these molecules. The foam surfactant produced by the protein skimmer was discarded daily and the skimmer cup cleaned. The venturi lines were flushed weekly with reverse osmosis water to prevent salt crystal build up which can cause a subsequent reduction of protein skimming efficiency.

The final section of the sump housed the main drive pump which supplied water to the broodstock aquarium via a 32mm unplasticized polyvinyl chloride (upvc) pipe (Fig 3.1 L). Branched off this were two 16mm hose valves which each fed a fluidised reactor (Fig 3.1 M) (Two Little Fishies – Phosban reactor 150) via 16mm silicone hose (www.advancefluidsolutions.co.uk). One reactor contained activated carbon (Vitalis, Carbonactive) for organic waste removal. The other reactor contained granulated ferric oxide (GFO) (ROWA Phos) that removed excess phosphates not taken up by the macro algae. Both carbon (300g) and GFO (500g) were replaced every two weeks and the old media discarded.

Each mesocosm aquarium was initially filled with a solar evaporated salt (H2Ocean Pro, D&D The Aquarium Solution), which was mixed in reverse osmosis (RO) water to a salinity closely matching that of the natal reef (Singapore 31.9 ppt and GBR 35 ppt). Salinity over the course

of the experiment was maintained (Singapore 32.59 ± 0.5 ppt and GBR 34.31 ± 1ppt) by automatic replacement of evaporative water with RO via 6 mm gravity fed supply line linked to a mechanical float. Following the initial fill, water chemistry within the mesocosm aquarium was managed following the Triton Method (https://www.triton.de/en/products-services/triton-method/). Four stock solutions (Triton, Core7) (Fig 3.1 N) were dosed to each mesocosm aquarium in equal proportions daily via a four-channel peristaltic pump (KAMOER KSP-F01A) (Fig 3.1 O). During the first month, the alkalinity of both mesocosm aquariums were measured daily (Salifert, AH/Alk Profi Test) and the dose rate adjusted to reach a target alkalinity of 7 dkh (2.5 meq/L). If alkalinity dropped, the dose rate of all stock solutions was increased until a dkh of 7 was stabilised. Water samples from each mesocosm aquarium were analysed monthly using inductively coupled plasma atomic emission spectroscopy (ICP-OES). The results indicated which element from the four stock solutions were absorbed by the corals and other biological processes within the mesocosm aquarium to a greater or lesser extent than the daily dose rate. Using a second four channel peristaltic pump, individual elements (Fig 3.1 P) were added to ensure that water chemistry parameters were maintained as close to natural seawater as possible (Appendix 4 for Singapore and Appendix 5 for Australia).

3.3.3 Environmental control

The seasonal environmental replication required to stimulate broadcast spawning was performed via a web-based microprocessor that contains a built-in web server (Neptune Systems, Apex) attached to each mesocosm aquarium. These consisted of a base unit (Fig 3.2 A), display module (Fig 3.2 B), energy bar (Fig 3.2 C), WXM Vortech/Radion wireless expansion module (Fig 3.2 D), and a lunar simulator module (LSM) (Fig 3.2 E). An IP address was assigned to the microprocessor for internet connection, via a router (NETGEAR 8 port 10/100 Mbps Switch FS608 v3) and Ethernet cable. Using the 'edit seasonal table' on the Apex classic dashboard (Fig 3.3), seasonal temperature, photoperiod and lunar cycle data were programmed for each study site. Sunrise, sunset, moonrise and moonset times were downloaded from www.timeanddate.com (Singapore and Cairns, the latter representing the GBR). For Singapore, annual variation in sea temperature was based on data collected during 2011 and 2012 using a data logger (Onset, HOBO Pendant temperature data logger UA-001-08) attached to the Kusu reef at approx. 3-4m (latitude 1.223874 longitude 103.862622). To generate the profile used in the mesocosm aquarium, the four daily measurements, taken every 6 hours, were averaged for the first day of each month. For the GBR mesocosm aquarium the temperature profile was generated from the Australian Institute of Marine Science (AIMS) online data centre's ten year average (2004-2014) temperature data set for Lizard Island (latitude -14.687517 longitude 145.4635) (http://data.aims.gov.au/aimsrtds/yearlytrends.xhtml).

Similarly, for the Singapore mesocosm aquarium the temperature value for the first day of each month was used to generate the GBR mesocosm profile. Additional water movement of 80,000 litre/hr was generated within the mesocosm aquariums by the use of four wave maker pumps (Jebao WR20) (Fig 3.1 Q), ensuring even temperatures throughout.

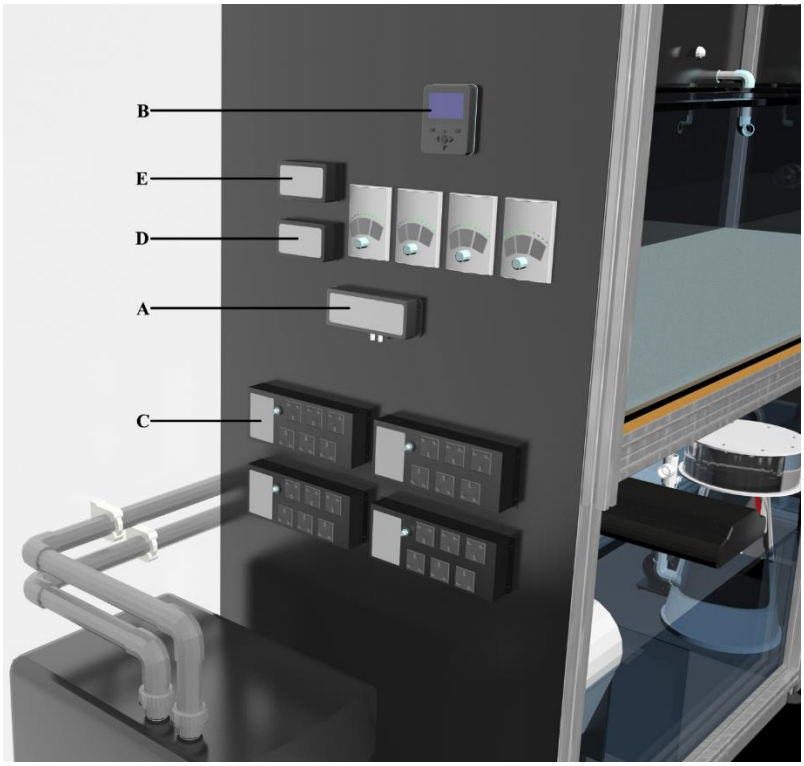


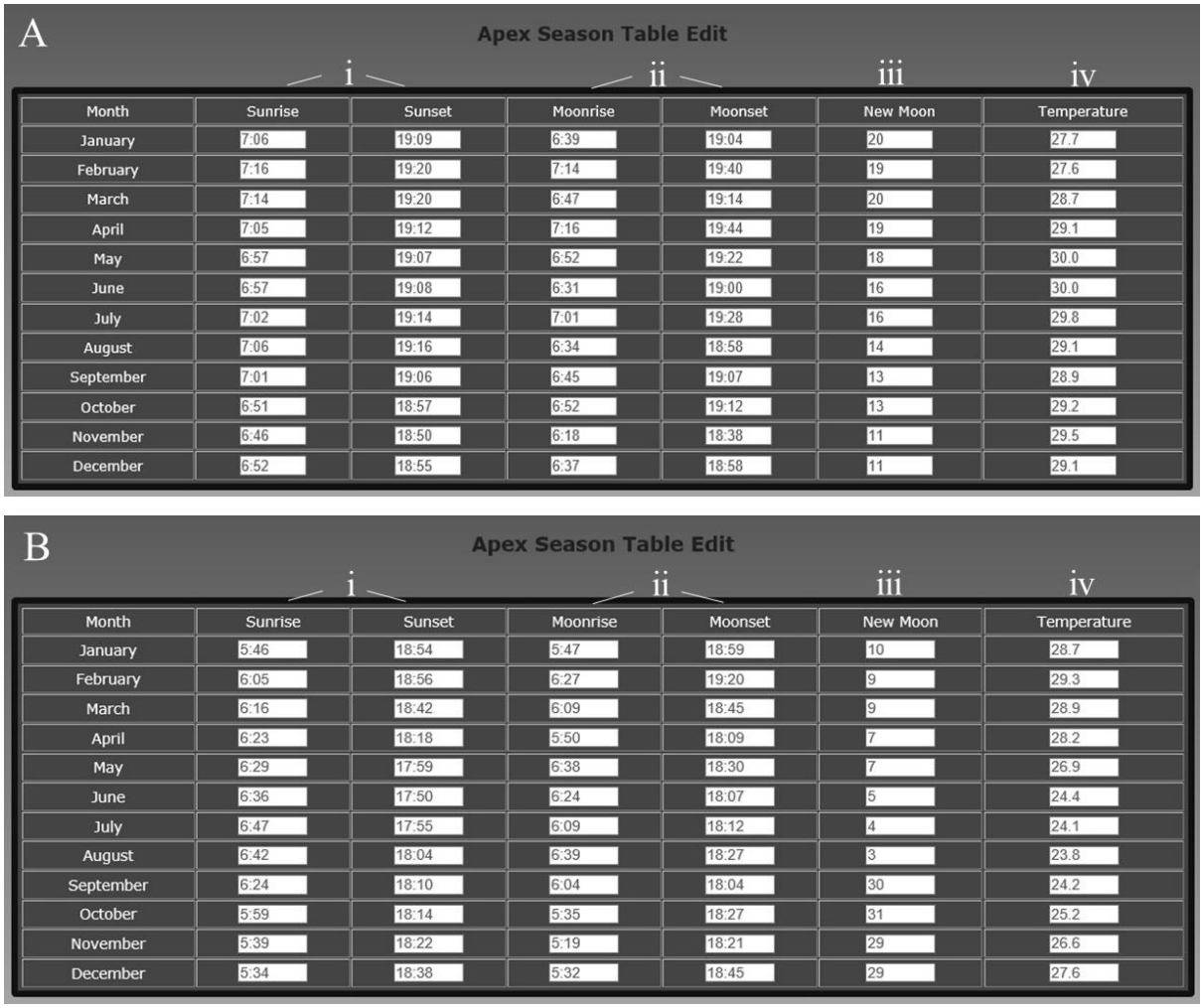
Figure 3. 2 Neptune Systems, Apex microprocessor to control environmental parameters within mesocosm.

(A) base unit; (B) display module; (C) energy bar; (D) WXM Radion wireless expansion module; (E) lunar simulator module.

3.3.4 Programming seasonal temperature replication

In order to replicate seasonal temperature change for each study site, the temperature value for the first day of each month was entered into each mesocosm aquarium seasonal table via the Apex classic dashboard. (Fig 3.3 A & B iv). The Apex averaged the temperature difference between each reading over the month creating a smooth curve throughout the year (Fig 3.4). Mesocosm aquarium water was warmed by three 300watt aquarium heaters (Visitherm) plugged into a power output on the energy bar (Fig 3.2 C). The corresponding output was then programmed (Appendix 6) to draw data from the seasonal table and turned the heaters on if the temperature fell below the daily set point. Conversely an aquarium chiller (Teco TR20) (Fig

3.1 R), programmed via a separate output (Appendix 6) turned on if the water temperature in the mesocosm aquarium required cooling.



A

Apex Season Table Edit

i ii iii iv

Month	Sunrise	Sunset	Moonrise	Moonset	New Moon	Temperature
January	7:06	19:09	6:39	19:04	20	27.7
February	7:16	19:20	7:14	19:40	19	27.6
March	7:14	19:20	6:47	19:14	20	28.7
April	7:05	19:12	7:16	19:44	19	29.1
May	6:57	19:07	6:52	19:22	18	30.0
June	6:57	19:08	6:31	19:00	16	30.0
July	7:02	19:14	7:01	19:28	16	29.8
August	7:06	19:16	6:34	18:58	14	29.1
September	7:01	19:06	6:45	19:07	13	28.9
October	6:51	18:57	6:52	19:12	13	29.2
November	6:46	18:50	6:18	18:38	11	29.5
December	6:52	18:55	6:37	18:58	11	29.1

B

Apex Season Table Edit

i ii iii iv

Month	Sunrise	Sunset	Moonrise	Moonset	New Moon	Temperature
January	5:46	18:54	5:47	18:59	10	28.7
February	6:05	18:56	6:27	19:20	9	29.3
March	6:16	18:42	6:09	18:45	9	28.9
April	6:23	18:18	5:50	18:09	7	28.2
May	6:29	17:59	6:38	18:30	7	26.9
June	6:36	17:50	6:24	18:07	5	24.4
July	6:47	17:55	6:09	18:12	4	24.1
August	6:42	18:04	6:39	18:27	3	23.8
September	6:24	18:10	6:04	18:04	30	24.2
October	5:59	18:14	5:35	18:27	31	25.2
November	5:39	18:22	5:19	18:21	29	26.6
December	5:34	18:38	5:32	18:45	29	27.6

Figure 3. 3 Apex seasonal table to programme annual photoperiod, lunar cycle and seasonal temperature change.

(A) Singapore 2015 seasonal table on the web-based Apex classic dashboard; (i) sunrise and sunset times for first day of each month, (ii) moonrise and moonset times for day of the month that new moon occurs, (iii) date of month that new moon occurs, (iv) annual temperature profile of Kusu reef, Singapore. Value entered for first day of each month; (B) GBR 2016 seasonal table on the Apex classic dashboard; (i) Sunrise and sunset times for Cairns on first day of each month, (ii) moonrise and moonset times for Cairns the on day of the month that new moon occurs, (iii) date of month that new moon occurs, (iv) annual

temperature profile derived from ten year average data set from Lizard Island, GBR. Value entered for first day of each month.

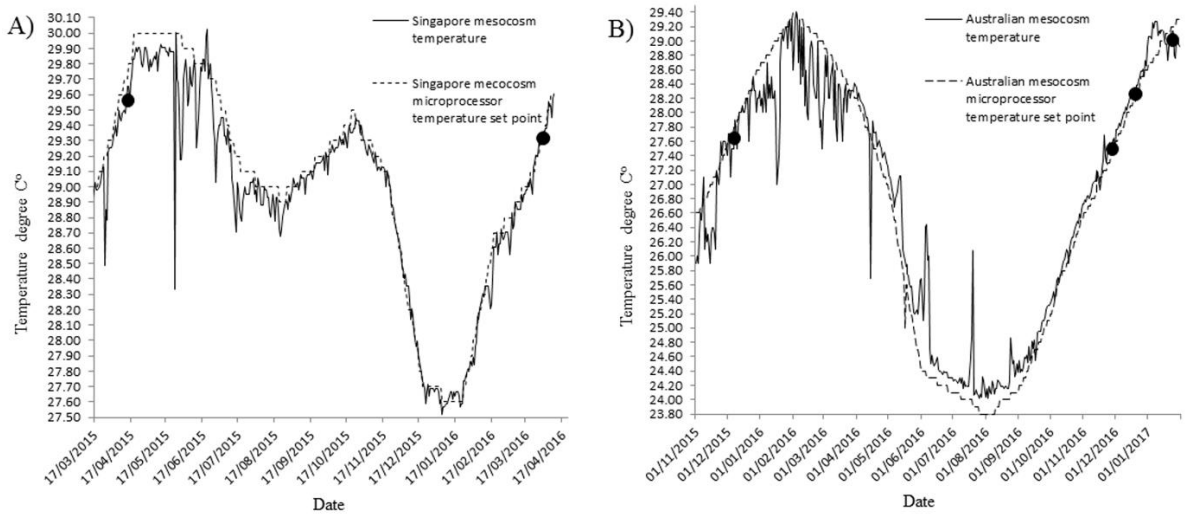


Figure 3. 4 Seasonal temperature change of Kusu Reef, Singapore and GBR, Australia showing averaged *in situ* and *ex situ* mesocosm profiles.

(A) Temperature profile of Singapore mesocosm replicating Kusu Reef. Dashed line represents temperature profile entered into the seasonal table, derived from data collected on Kusu reef between 2011 and 2012. Solid line – temperature of the mesocosm March 2015 to April 2016. (B) Temperature profile of *ex situ* mesocosm replicating GBR. Dashed line represents temperature profile entered into the seasonal table derived from AIMS ten year average temperature data set for Lizard Island. Solid line – temperature of the mesocosm from November 2015 to January 2017. ● denotes spawning events within the mesocosm.

3.3.5 Programming seasonal photoperiod and solar irradiance replication

Mounted on an extruded aluminium frame 30 cm above the mesocosm aquarium, eight Radion XR30w Pro LEDs (EcoTech Marine) (Fig 3.1 S) with wide angle lenses provided lighting for the corals. Each light was plugged into a separate power output on the energy bar and connected to the Apex through a WXM extension module via Wi-Fi. To simulate the sun's arc in the sky (from sunrise through to sunset) individual profiles were programmed through the classic dashboard. Three profiles were created, Rad_SunUp, Rad_Midday & Rad_SunDn (Appendix 7). The Radion's six LED channels (White, Blue, Royal Blue, Green, Red and UV) were set to

50, 100, 100, 50, 50 and 100% respectively. Rad_SunUp simulated a 3 hr increase in LED intensity starting at 0% at sunrise and ending at the appropriate intensity determined by the solar irradiance curve, detailed later. Rad_Midday simulated the midday solar intensity and defined the maximum power output of the LED. Rad_SunDn simulated a 3 hr ramp down from the midday intensity to 0% at sunset. Once these profiles were created each light was programmed via the WXM module (Appendix 8). In this way each light followed the photoperiod determined by the seasonal table (Fig 3.3) but incorporated an increase and decrease in intensity at the beginning and end of each day.

To replicate the annual shift in photoperiod the sunrise/sunset times for the first day of the month were programmed into the seasonal table for each location (Fig 3 A & B i). The Apex then calculated the appropriate time shift from one month to the next.

3.3.6 Solar irradiance

Whilst there is debate about the role that solar irradiance plays in driving spawning synchrony (van Woesik, Lacharmoise and Köksal, 2006; Keith *et al.*, 2016), it has been shown that solar irradiance correlates to egg maturation (Padilla-Gamiño *et al.*, 2014). In order to simulate this annual variation in photon intensity reaching the coral, 22 year irradiation averages from each study site were converted into data for LED programming. Using NASA Surface Meteorology and Solar Energy (https://eosweb.larc.nasa.gov/cgi-bin/sse/grid.cgi?email=skip@larv.nasa.gov) the GPS co-ordinates for each study location were entered and 22 year monthly average solar irradiance, in kWh m^{-2} d^{-1}, were downloaded. Annual solar irradiance curves were then generated by plotting solar intensity against month (Fig 3.5). Radion XR30w Pro % intensity was added to the secondary x axis starting at 60% (378µmol m^{-2} s^{-1}, ± 4), a value determined to be an appropriate low level intensity (Craggs per obs), increasing to 100% (498µmol m^{-2} s^{-1}, ± 10). A Radion XR30w Pro % intensity was then generated for each week through the year by drawing up from the y axis to the solar irradiance curve and then across to the secondary x axis. In this way a table of intensities was generated (Appendix 9). Each week the intensity of the three profiles were then changed to the appropriate week's % intensity (Appendix 7). In this manner solar irradiance curves from each study site were converted from NASA satellite data to *ex situ* LED lighting intensity.

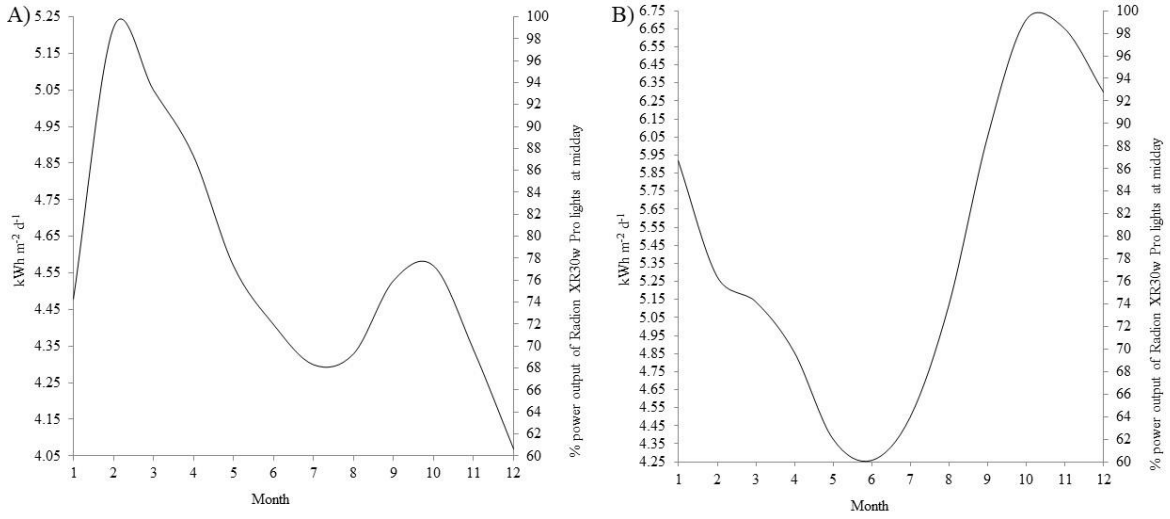


Figure 3. 5 22 year monthly average solar irradiance incident on a horizontal surface in kWh $m^{-2}d^{-1}$ at (A) Singapore; (B) Cairns, GBR.

3.3.7 Manipulation of spawning time

To ensure that spawning activity could be followed daily, spawning times were manipulated to occur during GMT daylight hours. In order to achieve this, clocks on each microprocessor were adjusted to move the time at which artificial sunset occurred in relation to GMT. In the Singapore mesocosm 12:00 Singapore time equated to 5:00 GMT. This ensured that *A. hyacinthus* would spawn between 14:00-15:00 GMT, equating to 21:00-22:00 Singapore time. 12:00 in the GBR mesocosm equated to 6:00 GMT which placed the predicted *A. tenuis* spawning window at 11:00-12:00 GMT and *A. millepora* and *A. microclados* 13:00-15:00 GMT, equating to 19:00-20:00 and 21:00-23:00 respectively (Australian Eastern Standard Time).

3.3.8 Lunar Cycle

The standard five LEDs that came with the Lunar Simulator Module (LSM) were modified replacing the blue spectrum LEDs with a kelvin temperature closely matching lunar light (4150K). Using a lux meter (Milwaukee MW700) the LED light intensity at 'full moon' was calibrated to 1 lux 1cm above the surface using half a spherical diffusing disc glued over each LED and tape to reduce light intensity (Fig 3.1 T). The LSM was then programmed via the classic dashboard (Appendix 10) reading from the seasonal table and through initial calibration, lunar phases were replicated. External light has been shown to influence spawning timing (Boch *et al.*, 2011; Vize, Hilton and Brady, 2012; Kaniewska *et al.*, 2015). Therefore, to prevent this disruption to spawning timing and synchrony with predicted wild dates, the Radion LED lighting rig was boxed-in on the sides, back and top with 5mm black MDF fitted into an aluminium frame (Fig 3.1 U). Integrated black-out blinds housed within the front of the aquarium framework (Fig 3.1 V) were then drawn 30 mins before sunset, facilitating the artificial control of the nocturnal light environment.

3.3.9 Heterotrophic feeding

One factor that has received little attention but may play an important proximate cue in both initiation of gamete production and subsequent development is the role of nutritional input via heterotrophy. A large amount of hermatypic scleractinian coral nutrition is derived from by-products received from their symbiont zooxanthellae or endolithic algae (Muscatine and Cernichiari, 1969; Fine and Loya, 2002). However many studies have shown scleractinian corals to be active heterotrophs, able to consume prey from bacteria to meta-zooplankton (Ferrier-Pagès *et al.*, 2003; Houlbrèque *et al.*, 2004; Ferrier-Pagès, Hoogenboom and Houlbrèque, 2011). Heterotrophy may account for the largest portion of the fixed carbon incorporated into skeletal structures and through prey capture up to 35% of a healthy coral's daily metabolic requirements can be met. This can increase to 100% during physiological stress, in particular during bleaching events associated with elevated SST (Fine and Loya, 2002). Houlbrèque & Ferrier-Pagès (2009) state that in addition to providing carbon, heterotrophy is likely to be important to most scleractinian corals since nitrogen, phosphorus, and other nutrients that cannot be supplied from photosynthesis by the coral's symbiotic algae must come from capture of plankton (picoplankton, phytoplankton and zooplankton),

particulate matter or dissolved compounds. Reproductive output represents a significant energy investment, with lipids playing a major role in reproductive tissue development compared to protein and carbohydrate (Leuzinger, Anthony and Willis, 2003). Leuzinger et al (2003) quantified lipid levels in pre- and post-spawning tissues and showed a decrease by 85 to 100% in a wide range of morphologically different scleractinian coral species as a result of spawning. Whilst there have been few studies focusing on the role of heterotrophy in relation to gamete production, Crook et al (2013) found a positive correlation to feeding and planula output in the brooding azooxanthellate scleractinian coral *Balanophyllia elegans*.

Although studies associating reproductive output and feeding are sparse, the fact that heterotrophy plays an important role in daily energetics of scleractinian corals makes it a reasonable assumption that nutrient input via heterotrophy plays an important proximate role in stimulating gamete production and subsequent development. In light of the ambiguity within the scientific literature of the potential role of heterotrophy the base assumption was made to provide a broad range of feed types to cover the potential positive input.

The filtration removed much of the naturally produced planktonic food within the mesocosm aquarium and therefore, to provide the carbon, nitrogen and phosphorus required for gamete production *ex situ* broodstock coral were fed daily. In addition, the broodstock aquarium was isolated from the filtration for 2 hrs per day to aid uptake, during which time the wave maker pumps remained on to provide water circulation. The following feeds were added to provide the variety of nutritional resources required by scleractinian corals: dissolved free amino acids, picoplankton, nanoplankton, microplankton and mesoplankton (Grover *et al.*, 2008; Houlbrèque and Ferrier-Pagès, 2009; Osinga *et al.*, 2011; Leal *et al.*, 2013). Amino acids, 0.02 ml/litre (AcroPower, Two Little Fishes), baker's yeast solution, 0.03ml/litre (1.75g baker's yeast, 14g sugar and 350 ml reverse osmosis water. This was freshly mixed three times each week in a conical flask, placed on a magnetic stirrer and incubated at 24ºC for 24 hrs prior to use. Solutions older than 72 hrs were discarded), 200 ml live *Tetraselmis spp*, 200 ml live *Artemia salina* nauplii (90 nauplii/litre), dead *Brachionus plicatilis* (8300/litre), fish eggs (2.4/litre), lobster eggs (5.8/litre) and cyclops (45.33/litre). Within 15 mins of feeding, colonies exhibited a positive response, evident from the expulsion of mesenterial filaments for prey capture (Fig 3.6) (Goreau, Goreau and Yonge, 1971; Goldberg, 2002; Wijgerde *et al.*, 2011).

At the end of each 2 hr isolation the water was clear of particles, indicating that the prey had been consumed.

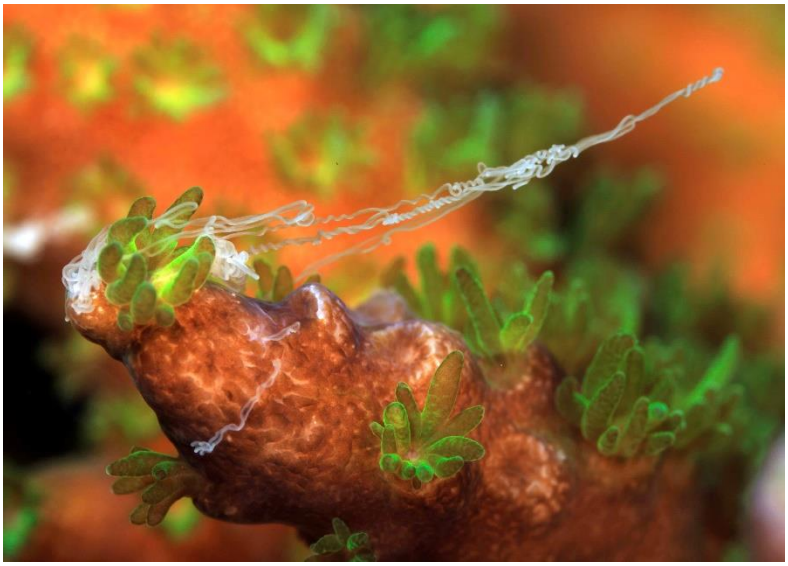


Figure 3. 6 Mesenterial filaments extended in *Acropora humilis* (not used within this study) in response to prey items added to the mesocosms during feeding isolation. *Images taken by J Craggs*

3.3.10 Control of algae and aquarium pests

Management of excessive algae growth and the control of pests that can cause harm to corals *ex situ* is an important husbandry component in running long term mesocosm experiments. To support these process various biological measures were utilised. In each mesocosm aquarium, one Yellow tang, Ze*brasoma flavescens*, one Convict tang, *Acanthurus triostegus*, one Foxfaced rabbitfish, *Siganus vulpinus*, and five Red legged hermit crabs, *Paguristes cadenati* were added to control turf algae growth. Fifteen Banded trochus snails, *Trochus spp* were used to manage cyanobacterial growth and four Tuxedo urchins, *Mespilia globulus* grazed crustose coralline algae. For pest management one Copperband butterflyfish, *Chelmon rostratus* controlled numbers of the sea anenome *Aiptasia spp* and one Silverbelly wrasse *Halichoeres leucoxanthus* was used to limit the population of red planaria *Convolutriloba retrogemma*.

3.3.11 Sampling for gamete development

Oocyte development within acroporids takes three to four months. Initial small white oocytes increase in size and eventually develop pigment, that can range from cream, orange and red, in the month prior to release (Wallace, 1985). Two months prior to the predicted wild spawning date for each study site, colonies were sampled for the presence of gametes to ascertain the stage of gamete development. Samples were taken between two and four days before the full moon and based on the oocyte development the expected *ex situ* spawning date of each colony was determined. Where possible, three branches per colony were fragmented making sure to avoid the infertile peripheral edge (Wallace, 1985). If the colony had insufficient branches a single branch was removed to prevent the colony reabsorbing oocytes as a result of colony stress (Okubo, Taniguchi and Motokawa, 2005). One sample set (between one and three fragments – see above) was taken the month following spawning to confirm that eggs had been released. Transverse sections were imaged (Fig 3.7) using a Canon 5d MKIII and MP-E 65mm lens set to ×5 magnification and illuminated using a Schott KL1500 LCD cold light source. Kelvin temperatures of light source and camera were matched (3300 Kelvin) to provide a true colour rendition. AH1–14 from Singapore were sampled on 1st February (Table 3.1 and Appendix 11), 26th February (Table 3.1 and Appendix 12), 17th March (Table 3.1 and Appendix 13) and 21st April 2016 (Table 3.1 and Appendix 14). Colonies AM1–5, AMIC1-6 and AT1-7 from GBR were sampled on 14th September (Table 3.2 and Appendix 15), 13th October (Table 3.2 and Appendix 16), 10th November (Table 3.2 and Appendix 17), 11th December 2016 (Table 3.2 and Appendix 18) and 8th January 2017 (Table 3.2 and Appendix 19). In addition colonies AM1 & 4 were sampled on 10th February 2017 as the gamete release from these individuals was delayed.

Table 3. 1 *Acropora hyacinthus* Singapore genotypes gamete sampling.

Colonies AH1 – 14 sampled on 1st February, 26th February, 17th March and 21st April 2016 for the presence or absence of oocytes pre and post *ex situ* spawning. Immature unpigmented oocytes (im), mature pigmented oocytes (m), oocytes not present (x)

Colony code	1st February 2016	26th February 2016	17th March 2016	21st April 2016
AH1	x	x	x	x
AH2	x	im	m	x
AH3	x	x	x	x
AH4	x	x	x	x
AH5	x	x	x	x
AH6	x	x	x	x
AH7	im	im	m	x
AH8	x	x	x	x
AH9	x	x	x	x
AH10	x	x	x	x
AH11	x	x	x	x
AH12	im	im	m	x
AH13	im	im	m	x
AH14	x	x	x	x

Table 3. 2 *Acropora millepora*, *A. microclados* and *A. tenuis*, GBR genotypes gamete sampling.

A.millepora (AM), *A. microclados* (AMIC) and *A. tenuis* (AT) sampled on 14th September, 13th October, 10th November, 11th December 2016 and 8th January 2017 for the presence or absence of oocytes pre and post *ex situ* spawning. Immature unpigmented oocytes (im), mature pigmented oocytes (m), oocytes not present (x)

Colony code	14th September 2016	13th October 2016	10th November 2016	11th December 2016	8th January 2017
AM1	im	im	im	im	m
AM2	im	im	im	m	x
AM3	im	im	m	x	x
AM4	im	im	im	im	m
AM5	im	im	im	m	x
AMIC1	im	im	m	x	x
AMIC2	im	im	im	m	x
AMIC3	im	im	m	x	x
AMIC4	x	x	m	x	x
AMIC5	im	im	m	x	x
AMIC6	im	im	m	x	x
AT1	im	im	m	x	x
AT2	x	x	x	x	x
AT3	im	im	im	m	x
AT4	x	x	x	x	x
AT5	x	x	x	x	x
AT6	x	x	x	x	x
AT7	im	im	im	m	x

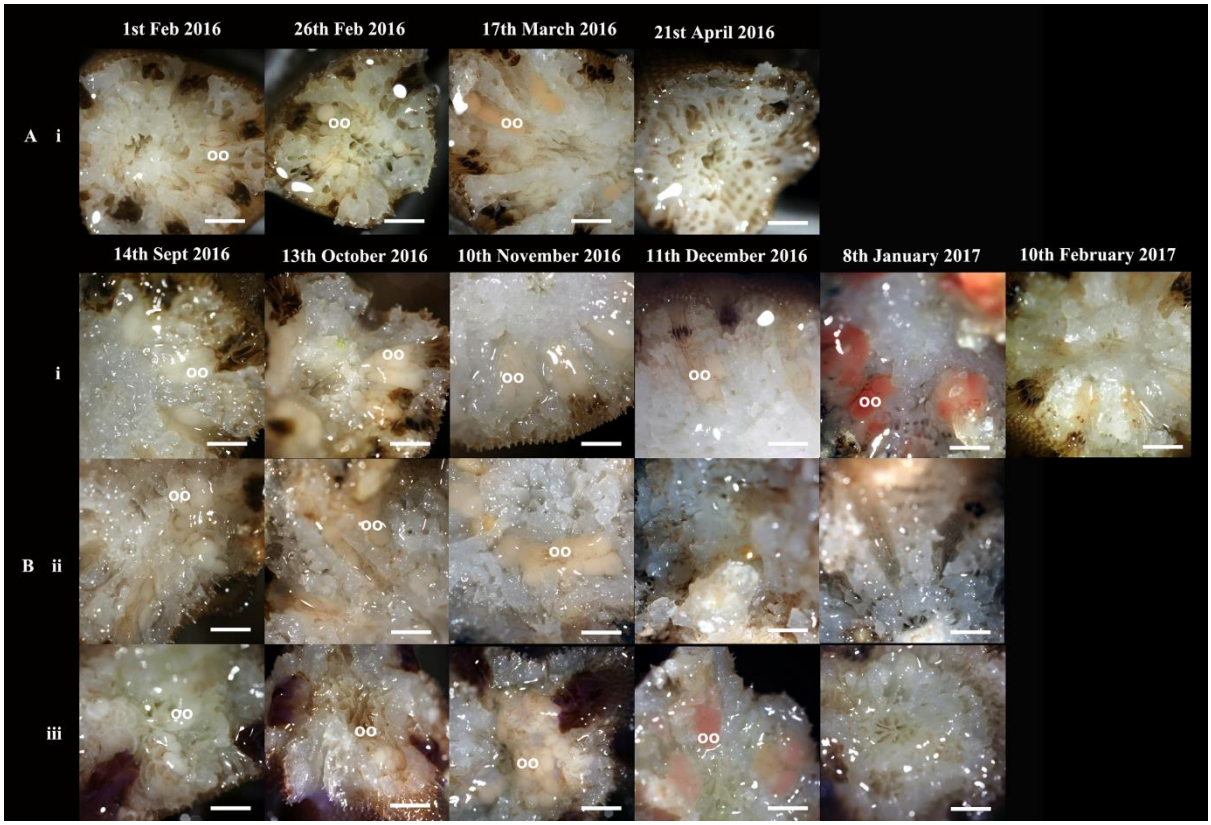


Figure 3. 7 Transverse sections of four species of *Acropora* showing polyps undergoing early and late-stage oocyte (oo) development in the build up to *ex situ* spawning.

(A) Singapore, (i) *Acropora hyacinthus*, showing AH12. (B) GBR, (i) *A. millepora*, showing AM1, (ii) *Acropora microclados*, showing AMIC5, (iii) *A. tenuis*, showing AT7. Scale 1mm. *All images taken by J Craggs*

3.3.12 Observing gamete release

Ex situ spawning activity was predicted based on the stage of oocyte development observed during sampling and the predicted wild spawning date for each location. *A. hyacinthus* in Singapore spawns between 20:00 and 22:00, three to five NAFM in March/April (Guest *et al.*, 2002). Colonies from the GBR spawn as follows: *A. tenuis* 00:10 to 01:15 (hours after sunset) three to six NAFM, Oct/Nov (Harrison *et al.*, 1984; Babcock *et al.*, 1986), and *A. millepora* 01:05 to 03:45 (hours after sunset) three to six NAFM, Oct/Nov (Harrison *et al.*, 1984; Babcock *et al.*, 1986). No reference to spawning activity was found for *A. microclados*, and so observations for this species followed those of *A. tenuis* and *A. millepora*. To ensure that any pre-spawn activity was recorded, observations started two NAFM on the predicted spawning

month. Observations continued daily up to 16 NAFM. One hour prior to the predicted spawning time the broodstock aquariums were isolated from the filtration by turning the main drive off. The four wave maker pumps were turned off 30 mins prior to the predicted spawning time leaving the water static. At this time floating gamete collecting rings were positioned directly above each gravid coral and held in place with clips. With no water movement present within the mesocosm aquariums, any released gametes floated directly upwards and were contained within the ring. These gamete collectors facilitated egg sperm collection and enabled genetic crosses to be made via in-vitro fertilisation. Following isolation from the sump, broodstock colonies were checked using red light torches every 15 mins for signs of bundle setting i.e. egg/sperm bundles in the mouths of the polyps (Edwards *et al.*, 2010). The broodstock aquariums remained isolated for three hours, ensuring that the spawning time window for each species had passed before the end of the isolation period. If no spawning occurred all pumps were turned back on, reconnecting the water flow from the filtration sump to the broodstock aquariums. Spawning times were recorded in terms of both artificial programmed time and real time GMT. Onset of spawning correlated with the observation of the first egg/sperm bundles being released.

Full moon occurred on 23rd March 2016 in the Singapore mesocosm aquarium and observations were conducted from 25th March to 4th April 2016. Observations in the GBR mesocosm aquarium spanned three months due to differences in spawning activity. Full moon 14th November 2016, observations from 16th to 30th November, full moon 14th December observations from 16th to 25th December 2016, full moon 12th January 2017 observations from 14th to 26th January 2017.

3.4 Results

3.4.1 Singapore spawning

On arrival in the UK it was noted that 10 out of 14 of the *Acropora hyacinthus* from Singapore were gravid and these spawned at 21:10 (14:10 GMT) between 10th and 13th April 2015, six to nine NAFM. Out of the original 14 colonies, four (28.57%) completed full gametogenic cycles during the experiment with spawning observed directly under a red light. Colony AH2 released

a pre-spawn, of a relatively few bundles, on 31st March 2016, eight NAFM. Colony AH2, 7, 12 and 13 released a full spawn on 2nd April 2016, 10 NAFM (Table 3.3). Spawning initiation was observed between 21:10 and 21:15 (14:10-14:15 GMT) and ceased between 21:35 and 21:42 (14:35-14:42 GMT). Wild spawning was predicted between three and five NAFM between 26th to 28th March and 25th to 27th April 2016 based on previous work (Guest *et al.*, 2002).

3.4.2 Australian spawning

At the point of arrival five out of five (i.e. 100%) of the *Acropora millepora* from the GBR, five out of seven (71.43%) of the *Acropora tenuis* from the GBR and three out of six (50%) of the *Acropora microclados* from the GBR were gravid. These spawned between 19:18 (11:18) and 21:17 (13:17) between 2nd and 7th December 2015, 6 and 11 NAFM (Table 3.3).

All three species of *Acropora* from GBR completed full gametogenic cycles during the experiment (100% of *A. millepora,* 100% *A. microclados* and 57.14% *A. tenuis*, n = 5, 6 & 7), with spawning extending over a period of three months (November 2016 – January 2017). Direct observations were made in all three species (colony numbers: AT3, AT7, AM2, AM4, AM5, AMIC2 and AMIC3) (Fig 3.8) with spawning occurring between 14 and 16 NAFM November 2016, 6 and 14 NAFM December 2016 and 9 and 14 NAFM January 2017. Onset times of spawning for *A. tenuis, A. millepora* and *A. microclados* were 19:26-19:32 (11:26-11:32 GMT), 21:06-21:49 (13:06-13:49 GMT) and 22:10-22:30 (14:10-14:30 GMT) respectively.

Where spawning was not directly observed, gamete release was inferred by the absence of oocytes during sequential sampling. Spawning observation at the National Sea Simulator (SeaSim) at AIMS was used as a proxy for the wild spawning time periods. Here *A. tenuis* and *A. millepora* spawned between 3 and 7 NAFM on 17th and 21st November 2016. No comparison for *A. microclados* wild spawning was available.

One colony, AT5, exhibited symptoms consistent with white syndrome (Fig 3.9) (Sweet *et al.*, 2013) and subsequently did not spawn. It is possible that the onset of this was a result of the mesocosm in which this colony was housed being isolated around spawning time leading to a reduction of oxygen levels.

Figure 3. 8 *Acropora millepora* releasing egg-sperm bundles following induced spawning *ex situ*.

Image taken by J Craggs

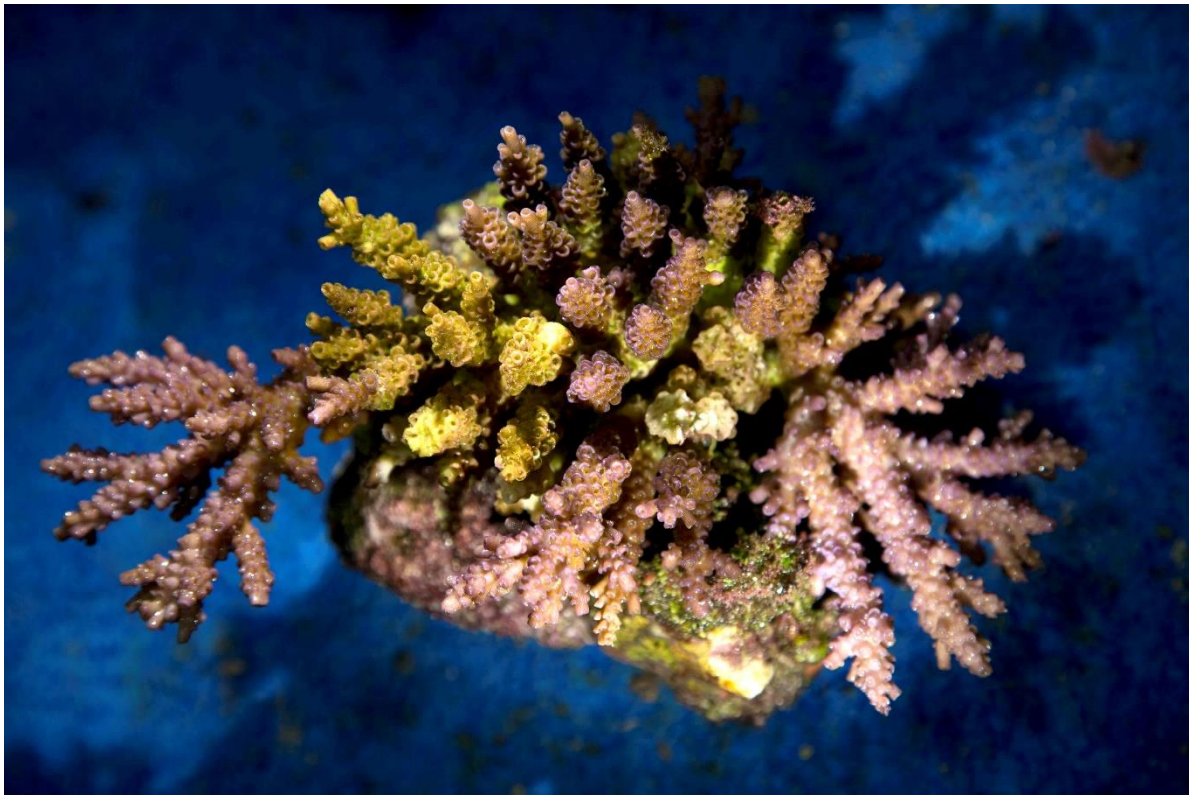


Figure 3. 9 *Acropora tenuis* (AT5) exhibited symptoms consistent with white syndrome. *Image taken by J Craggs*

Table 3. 3 *In situ* and *ex situ* spawning observations for four *Acropora* species during 2015 and 2016.

Acropora hyacinthus in Singapore mesocosm. *Acropora millepora*, *Acropora tenuis* and *Acropora microclados* in GBR mesocosm. First egg/sperm bundle release denoted spawn start time. *Ex situ* spawn times recorded at time relating to artificial light cycle time based on microprocessor programming and as GMT. NAFM denotes the number of nights after full moon that spawning occurred.

	2015					2016			
Species	Wild Spawning	Colony code	Date of spawning	NAFM	Spawning start time in relation to artificial sun set (GMT)	Wild spawning	Date of spawning	NAFM	Spawning start time in relation to artificial sun set (GMT)
		AH1	12/04/2015	8			Colony not gravid		
A. hyacinthus	4/4/15 4 NAFM Spawning start time 21.00	AH2	12/04/2015	8		26/3/16 - 28/3/16 3-5 NAFM Spawning start time 21.00	31/03/2016	8	21:11 (14:11)
							02/04/2016	10	21:14 (14:14)
		AH3	Colony not gravid				Colony not gravid		
		AH4	12/04/2015	8			Colony not gravid		
		AH5	12/04/2015	8			Colony not gravid		
		AH6	12/04/2015	8			Colony not gravid		
		AH7	12/04/2015	8			02/04/2016	10	21:11 (14:11)
		AH8	12/04/2015	8			Colony not gravid		
		AH9	09/04/2015	5	21:10 (14:10)		Colony not gravid		
		AH10	12/04/2015	8			Colony not gravid		
		AH11	Colony not gravid				Colony not gravid		
		AH12	12/04/2015	8			02/04/2016	10	21:12 (14:12)
		AH13	12/04/2015	8			02/04/2016	10	21:10 (14:10)
		AH14	12/04/2015	8			Colony not gravid		
A. millepora	28/11/15 - 4/12/15 2-8 NAFM Spawning time 21.25	AM1	02/12/2015	6	21:15 (13:15)	19/11/16- 21/11/16 5-7 NAFM Spawning start time 20.40	Spawning inferred between 8th January 2017 and 10th February 2017		
		AM2	02/12/2015	6	21:17 (13:17)		25/01/2017	13	21:49 (13:49)
		AM3	Spawning inferred between 20th November and 21st December				Spawning inferred between 10th November and 11th December 2016		
		AM4	07/12/2015	11	21:09 (13:09)		29/11/2016	15	21:07 (13:07)
							30/11/2016	16	21:06 (13:06)
							21/01/2017	9	21:20 (13:20)
							22/01/2017	10	21:15 (13:15)
							23/01/2017	11	21:18 (13:18)
							24/01/2017	12	21:15 (13:15)
							25/01/2017	13	21:09 (13:09)

		AM5	06/12/2015	10	21:09 (13:09)		22/01/2017	10	21:15 (13:15)
			07/12/2015	11	21:06 (13:06)		23/01/2017	11	21:15 (13:15)
							26/01/2017	14	21:21 (13:21)
A. tenuis	30/11/15 - 1/12/15 4-6 NAFM Spawning time 19.00-19.30	AT1	Spawning inferred between 20th November and 21st December			17/11/16 - 21/11/16 3-7 NAFM Spawning time 19.10-19.30	Spawning inferred between 10th November and 11th December 2016		
		AT3	06/12/2015	10	19:18 (11:18)		22/12/2016	8	19:32 (11:32)
							23/12/2016	9	19:26 (11:26)
		AT5	07/12/2015	11	19:20 (11:20)		White syndrome outbreak. Did not spawn		
		AT7	02/12/2015	6	19:20 (11:20)		20/12/2016	6	19:30 (11:30)
			03/12/2015	7	19:19 (11:19)		21/12/2016	7	19:32 (11:32)
			04/12/2015	8	19:19 (11:19)		22/12/2016	8	19:30 (11:30)
A. microclados	No data available for the species	AMIC1	Spawning inferred between 20th November and 21st December			No data available the species	Spawning inferred between 11th December 2016 and 8th January 2017		
		AMIC2	Colony not gravid				28/12/2016	14	22:30 (14:30)
		AMIC3	Spawning inferred between 20th November and 21st December				28/11/2016	14	22:10 (14:10)
		AMIC4	Spawning inferred between 20th November and 21st December				Spawning inferred between 10th November and 11th December 2016		
		AMIC5	Colony not gravid				Spawning inferred between 10th November and 11th December 2016		
		AMIC6	Colony not gravid				Spawning inferred between 10th November and 11th December 2016		

3.5 Discussion

Despite over three decades of research into broadcast spawning biology in reef building corals, to the best of my knowledge, there have been no successful attempts (to date) to maintain ambient environmental conditions and natural spawning rhythms of any broadcast spawning coral in closed system mesocosm aquaria over a full annual gametogenic cycle. All four species used in this experiment completed full gametogenic cycles. Spawning times post sunset for *Acropora hyacinthus*, *Acropora millepora* and *Acropora tenuis* were consistent with time windows observed in the wild (Harrison *et al.*, 1984; Babcock *et al.*, 1986; Guest *et al.*, 2002), a result indicating that the influence of the diel cycle associated with spawning time was maintained in these colonies for a period of over one year. In contrast, spawning times in relation to the lunar cycle were delayed in most colonies and occurred up to nine nights later than expected. While the integrated black-out system was designed to reduce external light influences and allowed me to manipulate the spawning to occur during daylight hours GMT, the resulting light pollution possibly affected gene regulation that interfered with chemical signalling pathways responsible for inducing gamete release and may, at least in part, explain these observed shifts (Boch *et al.*, 2011; Vize, Hilton and Brady, 2012; Kaniewska *et al.*, 2015).

The variations seen in the percentage of colonies developing eggs (28.57% in *A. hyacinthus*, 100% in *A. millepora* and *Acropora microclados* and 57.14% in *A. tenuis*) reflects those observed in wild populations (Guest *et al.*, 2005). However, it is possible that with improved heterotrophic nutrition the percentage of colonies successfully spawning in mesocosms could be increased. A study by Séré et al. (2010) explored this possibility but found no positive correlation with heterotrophic input and improved gamete output. However, the experimental configuration only utilised one food source (rotifers) and this is likely to have under-represented the range of nutrients needed by corals to sustain energy demanding processes such as reproduction and spawning. Further research is therefore needed to confirm if the use of heterotrophic feeding can be harnessed to increase reproductive success and output.

This study aimed to design a mesocosm aquarium that simulated the natural environment as accurately as possible. The objective was to simply close the reproductive cycle of these corals *ex situ* which if successful, would then enable researchers to start to disentangle environmental variables, such as; thermal shifts as a result of currents and weather patterns, changes in photoperiod, and solar irradiance and lunar light intensity. In turn, this would allow for the assessment of the roles each of these variables play in reproduction in these organisms as a

whole. It is likely that there is no single variable which induces gamete production and spawning in these corals. However, now we are able to manipulate these parameters in a controlled setting in order to assess the effect these have (singly and with potential interactions) on the end result.

Furthermore, the design and success of this study allows researchers to produce large numbers of coral larvae and juveniles for other experiments in a much wider range of locations than was previously possible. Such experiments could focus on larval settlement (Nishikawa and Sakai, 2005), along with assessing the impacts of climate driven thermal stress (Nozawa and Harrison, 2007) or ocean acidification on early ontogeny (Albright *et al.*, 2010). We are now also able to experiment with selective egg and sperm crosses from different colonies or between species in order to assess drivers of survivorship and to understand the pathways of genetic inheritance. In addition, such a breakthrough in coral rearing i.e., the successful *ex situ* spawning and ability to genetically select for and cross specific genotypes offers great possibilities for researchers interested in the possibility of human assisted evolution (van Oppen *et al.*, 2015). In this regard, we can now assess how, or even what affect hybridisation may have on the evolution of reefs, including but not limited to range expansion and adaptations to changing environmental conditions (Willis *et al.*, 2006; van Oppen *et al.*, 2014). Current research associated with broadcast spawning has a limited window of time in which material is available from wild spawning events (Harrison *et al.*, 1984; Okubo and Motokawa, 2007; van Oppen *et al.*, 2014; Teo *et al.*, 2016). However, the successful *ex situ* manipulation of environmental parameters may now, allow us to facilitate spawning events that break these natural spawning rhythms, a result which will ultimately lead to the possibility of year-round broadcast reproductive events. The increase in access to material that this would support could provide a significant platform to accelerate our understanding in the aforementioned research areas. Finally, the up scaling of *ex situ* mesocosm aquarium systems as reported here, has the potential to support large scale coral reef restoration efforts by increasing the frequency at which genetically diverse coral larvae are available for transplantation.

Chapter 4: Embryogenesis of reef building corals following *ex situ* induced gametogenesis and subsequent broadcast spawning

4.1 Abstract

Documentation of embryogenesis in broadcast spawning corals provides valuable baseline information of early ontogeny. This facilitates research into broader scale observations of deleterious effects of anthropogenic influences on coral reproduction, such as pollution and thermal stress, and can provide insights into how these influences may impact coral reefs on a global scale. *Acropora* is the most widely spread and abundant reef building coral genus, but it is only recently that broadcast spawning of this group (see Chapter 3) has been successfully induced in fully closed *ex situ* mesocosms. As such no study has yet investigated the potential effects, positive or negative, such *ex situ* spawning can have on embryological development. In this Chapter, I therefore documented the embryogenesis of two *Acropora* species (*Acropora tenuis* and *Acropora millepora*) that completed full gametogenic cycles *ex situ* and a third species (*Acropora anthocercis*) in which colonies were imported one month prior to spawning. Utilising scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) techniques, coupled with spawning behavioural characteristics, I illustrate that embryological stages of development and the rate of cellular division in all three species were consistent with those previously described *in situ*. Significant differences in egg/sperm ascending speeds and bundle dissociation times were also observed between the three species, with *A. anthocercis* bundles ascending more rapidly but with protracted bundle dissociation times. Such observations may provide insights into reproductive adaptations, including fine scale understanding of prezygotic isolation barriers, that may have evolved in response to ecological zonation and the environmental conditions experienced therein. Finally, I explored if oocyte size could provide an indication of gamete quality. Oocytes released from *A. tenuis* and *A. millepora* were smaller (396 ± 22µm and 385 ± 36µm (mean ± SD) respectively) than those previously recorded for the same species. A result which may indicate a possible negative effect on the quality of gametes developing *ex situ*. However, fertilisation rates remained high for all three species (>95%) suggesting no such effect occurred. CLSM images of fluorescent marked sperm DNA indicated that sperm aggregations were uniformly distributed over the oocyte during fertilisation, with no polarity of sperm entry site being observed. In addition

SEM photomicrographs of preserved cells revealed no developmental abnormalities as a result of *ex situ* gamete development. I conclude that the quality of gametes and embryos resulting from *ex situ* spawning is comparable to spawn and larvae collected directly from reefs. These results suggest that *ex situ* reproduction provides new and diverse research opportunities for the future.

4.2 Introduction

Coral reefs are facing unprecedented levels of pressure as a result of anthropogenic influences (Hoegh-Guldberg, 2011; Hughes, Barnes, *et al.*, 2017). These drivers are leading to increasing levels of thermal stress causing pan global coral bleaching events (Hughes *et al.*, 2017), acidifying of the oceans (Hoegh-Guldberg *et al.*, 2007), pollution (Kennedy *et al.*, 2013), sedimentation (Bartley *et al.*, 2014), and higher levels of disease (Peters, 2015). Reproduction is a fundamental process in all aspects of life, one that ensures population persistence. Therefore, understanding the early life stages of organisms such as corals, including behaviour, gamete and embryological development, has therefore received considerable research attention over the past 40 years (Babcock and Heyward, 1986; Okubo and Motokawa, 2007; Okubo *et al.*, 2013). This research has already provided important insights into the fundamentals of reproductive biology and evolutionary pathways (Miller and Ball, 2000; Ball *et al.*, 2002; Ying *et al.*, 2018). Developing an even greater understanding of embryogenesis support the study of broader aspects of coral biology such as possible negative impacts of climate change (Negri, Marshall and Heyward, 2007; Portune *et al.*, 2010), sedimentation (Jones, Ricardo and Negri, 2015; Ricardo *et al.*, 2015) and pollution (Negri and Heyward, 2001; Humanes *et al.*, 2016) on coral populations. Various methods are utilised to study gametes including histology (Babcock and Heyward, 1986), scanning electron microscopy (SEM) (Miller and Ball, 2000; Chui *et al.*, 2014), fluorescent *in situ* hybridisation (Sharp *et al.*, 2010) and confocal laser scanning microscopy (CLSM) (Marlow and Martindale, 2007). To date, these studies have relied on the collection of gametes from *in situ* spawning events. It is only relatively recently that gametogenic cycles and subsequent release of broadcast spawning gamete in *Acropora* species have been successfully induced in a fully closed *ex situ* mesocosm (Craggs et al. 2017, Chapter 3). These recent advancements can facilitate further research on coral reproductive and support restoration efforts by increasing the access to gametes throughout the year as opposed to the limited windows of time during periodic, short wild broadcast spawning events (Harrison *et*

al., 1984; Negri *et al.*, 2001; Humphrey *et al.*, 2008). Further, such controlled *ex situ* spawning negates the need for institutions to be located in close proximity to tropical reefs, considerably increasing the global number of institutions with the potential to contribute to the research field of coral reproduction.

Gamete development and spawning are just one part of the reproductive process (Jones, Ricardo and Negri, 2015). For corals to recruit into a reef system, or be viable for research experimentation, fertilisation and subsequent embryogenesis must also be successful.

Utilising the new potential in *ex situ* spawning, we do not currently understand the potential implications (positive or negative) for embryo development as a result of broodstock colonies being maintained *ex situ* and spawning within these mesocosms. I document in this Chapter the fertilisation and cellular division which occurs during the early ontogeny of three complex reef building broadcast spawning species, *Acropora tenuis, Acropora millepora* and *Acropora anthocercis.* This was undertaken using SEM and CLSM and aimed to investigate whether *ex situ* care influences embryological development.

4.3 Methods

Three reef building *Acropora* species, *A. tenuis*, *A. millepora* and *A. anthocercis* from the Great Barrier Reef were utilised in this study. The ecological characteristics of the collection sites for the three species varied and are described by the collection divers as follows. Colonies of *A. anthocercis* were distributed in shallow water (1-5m) on exposed front reefs of the outer barrier reefs, in high current. *A. millepora* were predominately found in shallow (1-4 m) surface reefs on the middle barrier reefs. These can reside on the front or back of the reefs, with low protection from medium to high currents. *A. tenuis* colonies inhabited partially protected back edges of the middle barrier reefs (5-12 m) in low to medium currents.

Five *A. millepora* and seven *A. tenuis* separate genotype colony fragments (diameter: <20cm) were transported from the Great Barrier Reef (GBR) (CITES import permit number: 537547/02) in 2015. These spawned in December 2015 (Chapter 3) and then completed full gametogenic cycles *ex situ* during the following 12 months. Branches were removed monthly from Sept – Dec, a few days prior to full moon, from each colony and cross sections of the exposed skeleton imaged to document gamete development and predict spawning date (Fig

4.1). In addition six colony fragments of *A. anthocercis* were transported (CITES import permit number: 545320/03) one month prior to the predicted wild spawning date (Babcock, Willis and Simpson, 1994). All colony fragments were housed in a closed system mesocosm aquaria at the Horniman Museum and Gardens, London, and maintained following methodology described previously by Craggs et al. (2017) and Chapter 3. Artificial full moon occurred on 14th November 2016. Previous studies (Craggs *et al.*, 2017) show a three/four night delay in gamete release occurs *ex situ* compared to the wild. Therefore, starting five NAFM, 30 mins prior to predicted spawning time, *A. tenuis* 19.00-20.00, *A. millepora* 21.00-22.30 (Babcock *et al.*, 1986) and *A. anthocercis* 22.50 (Babcock, Willis and Simpson, 1994) the mesocosm aquarium pumps were turned off, leaving the water static. In the absence of water movement, due to the high concentration of buoyant wax esters within the oocytes (Arai *et al.*, 1993; Padilla-Gamiño *et al.*, 2013), the oocyte/sperm bundles migrated vertically upwards into the gamete collectors. To record egg/sperm bundle ascending rates a ruler was placed vertically adjacent to the colony (Appendix 20) and the spawn from each species filmed (Canon 5D Mark III with 100 macro lens). Videos were analysed and bundle ascending speeds calculated (Table 4.1 C). Two *A. tenuis* colonies spawned on 21st November 2016, 7 NAFM (Table 4.1 A & B). Four *A. millepora* and four *A. anthocercis* colonies spawned on 23rd November 2016, 9 NAFM (Table 4.1 A & B). Immediately following gamete release three aliquots containing approximately 50 egg/sperm bundles were skimmed from the surface into 50ml Falcon tubes and each topped to 45 ml with newly mixed sea water. In order to standardise agitation the tubes were placed onto a blood rotator (Maplelab Scientific, RM-3) at 10 rpm (Appendix 21) and the time taken for all bundles to disassociated in each sample was recorded (Table 4.1D).

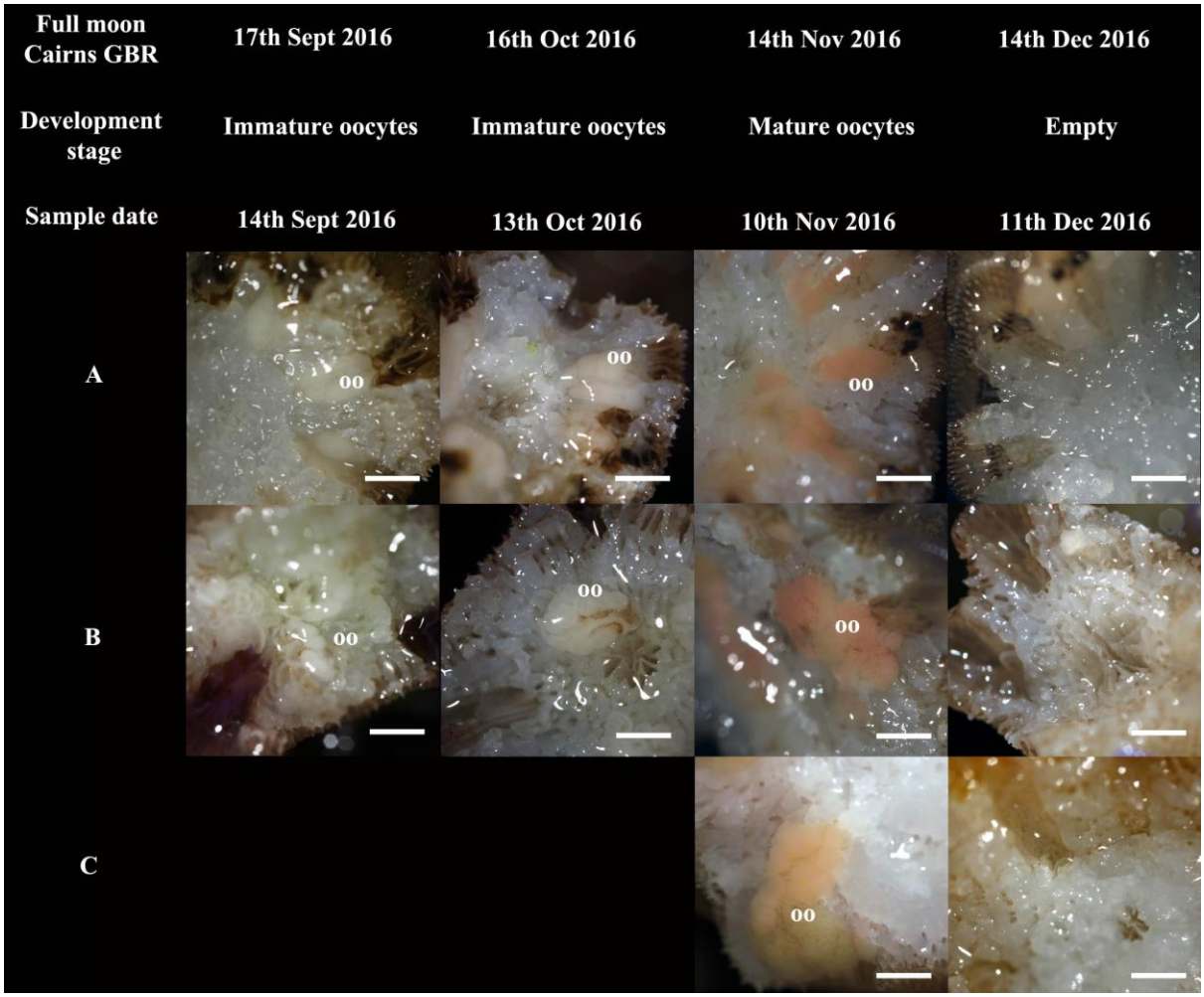


Figure 4. 1 Transverse sections of three species of *Acropora* showing polyps undergoing early and late-stage oocyte development in the build up to *ex situ* spawning.

(A) *Acropora millepora* completing full gametogenic cycle *ex situ*; (B) *Acropora tenuis* completing full gametogenic cycle *ex situ*; (C) *Acropora anthocercis* imported gravid one month prior to predicted spawning. oo = oocytes. Scale 1mm. *All images taken by J Craggs*

Preceding gamete separation, pure species crosses were made following *in vitro* fertilisation techniques described by Guest et al (2010) as a chapter in (Edwards *et al.*, 2010). Oocytes and sperm were mixed for 45 mins prior to sperm being siphoned off, and oocytes were washed three times in newly mixed seawater. To quantify fertilisation rates, percent success of three samples (of >50 embryos per cross) were taken 2 hrs post fertilisation and imaged using Canon 5d MKIII and MP-E 65mm lens, set to ×5 magnification and illuminated using a Schott KL1500 LCD cold light source. Ratios of fertilised two cell blastomeres to unfertilised oocytes were counted using Image J (Schneider, Rasband and Eliceiri, 2012) and percentage fertilisation success calculated (Table 4.1 C)

4.3.1 Mapping embryogenesis with Scanning Electron Microscopy (SEM)

To document the embryogenesis of all three species, aliquots from each cross were taken at set points. Egg/sperm bundles and bundles disassociating were taken during release, *in vitro* samples were taken every 15 mins during the first 45 mins of fertilisation and embryological samples taken hourly during the first 12 hrs after fertilisation (T = 0 to T = 12), every 2 hrs from T = 12–24, every 4 hrs from T = 24–72, and every 12 hrs thereafter. Samples were fixed in 4% glutaraldehyde (GA) for 24 hrs at 8 ^{o}C, followed by three 0.2M phosphate buffered saline (PBS) washes. Sample dehydration was performed in serial ethanol dilutions (30, 50, and 70%) with two washes 15 mins apart at each concentration and stored at 8 ^{o}C until further processing. To prepare samples for SEM a further serial ethanol dilution (80, 90, 95, 100% with a final step of a future 100% with a molecular sieve) was undertaken. Tissue samples were critical point dried (Balzers CPD 030), mounted on aluminium stubs with double sided conductive carbon tape and ion sputter coated with gold/palladium to 20 mm thickness (Cressington sputter coater, 208 HR). Samples were viewed and photographed with SEM (Zeiss Ultra Plus) at 3.00kV.

4.3.2 Confocal Laser Scanning Microscopy (CLSM)

To determine the sperm entrance sites into the oocyte (during fertilisation), aliquots of *A. tenuis* and *A. millepora* gametes/embryos were taken during bundle release, bundle dissociation, every 15 mins for the first 45 mins of fertilisation and then 1 and 2 hrs post fertilisation. Samples were preserved in 4% paraformaldehyde in phosphate buffered saline and 14% sucrose at 5 ^{o}C for 24 hrs. Following fixation, samples were washed three times in a PBS and 14% sucrose mix. Sperm nuclei were counterstained with 300nM SlowFade® Gold Antifade Mountant with DAPI (ThermoFisher Scientific, S36938) for 5 mins at room temperature, followed by three PBS rinses.

To determine the location of sperm clusters in relation to oocyte nuclei location, a subset of each sample was histologically processed and 5 µm serial sections slide mounted. Following processing mounted samples were marked with DAPI (as above) to highlight the nucleus, and sperm microtubules were labelled with Alpha Tubulin Mouse Monoclonal Antibody (ThermoFisher Scientific, 62204) at 2 µg/mL in 0.1% BSA and incubated for 3 hrs at room temperature and then labelled with Goat anti-Mouse IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 488 conjugate (ThermoFisher Scientific, A28175), a dilution of

1:2000 for 45 mins, also at room temperature. Finally actin cytoskeleton was stained with Alexa Fluor® 555 Rhodamine Phalloidin (ThermoFisher Scientific, R415) at 1:300 dilution in PBS with 1% bovine serum albumin (BSA) to reduce nonspecific background staining.

Samples were then scanned using a Nikon A1-Si confocal laser scanning microscope (CLSM) (Nikon Corporation, Tokyo, Japan) fitted to a Nikon Eclipse upright microscope. Four laser channels (blue channel = DAPI, green channel = Fluorescien, orange channel = JOJO and red channel = Alexa Fluor 64) with excitation wavelengths of 403, 487, 561 and 638nm respectively were used, with a pinhole aperture of 29.4 µm, to produce Z stack images.

4.4 Data analysis

Linear regression models (LM) were used to assess egg/sperm bundle ascending speed, flowing gamete release, bundle dissociation time and percentage fertilisation success as a function of species (treated as a fixed factor). Assumptions of homogeneity and normality were assessed using residual diagnostics, and Cook's distances were used to identify no overly influential data points following Zuur et al. (2007). Model assumptions held and there were no overly influential data points. The analyses were conducted using the statistical programming language R (R Development Core Team, 2015) R version 3.3.0 (2016-05-03).

4.5 Results

In total, ten colonies across the three species spawned, with *Acropora tenuis* releasing gametes two nights prior to *Acropora millepora* and *Acropora anthocercis* (Table 4.1 A). The spawning times of *A. tenuis* and *A. millepora* were consistent with that reported in the wild (Table 4.1 B) (Babcock *et al.*, 1986). There is no record of the spawning time for the third species, *A. anthocercis* (on the GBR). During my study spawning occurred between 22:49-23:20 (Table 4.1B).

There were significant differences in the rates of ascent of egg/sperm bundle amoung the three species. Those released from *A. anthocercis* were more buoyant (Table 4.1 C), with significantly greater ascending speeds compared to both *A. millepora* and *A. tenuis* ($p<0.001$, & $p<0.001$ respectively) (Table 4.2 A, Fig 4.2 A). Ascending speeds for *A. millepora* and *A. tenuis* were not significant from one another ($p=0.683$).

Following egg/sperm bundle release, sulphated mucosubstances that keep the bundle together (Okubo and Motokawa, 2007) were observed via SEM (Fig 4.3 A&B). Fluorescently marked sperm DNA indicated that liberation from the bundle in *A. millepora* commences within the first 10 mins following bundle release (Fig 4.4 A). Further, as dissociation progresses, increasing numbers are released (Fig 4.3 C&D). Interestingly, significant differences associated with the speed of dissociation occurred between all three species (p=<0.001) (Table 4.2 B, Fig 4.3 B), with the egg/sperm bundle breaking apart fastest in *A. tenuis* (23 ± 1.73 mins mean± SD), followed by *A. millepora* (46.67 ± 4.16 mins, mean ± SD) (Table 4.1 D). For *A. anthocercis,* bundle dissociation time was protracted (137 ± 6.24 mins, mean ± SD), taking three and six times longer than *A. millepora and A. tenuis* respectively (Table 4.1 D).

Table 4. 1 Comparison of *Acropora anthocercis, Acropora millepora and Acropora tenuis* gamete spawning ecology.

(A) Night indicates the number of nights after full moon; (B) Spawning time ranged from 19.20–23.20; (C) Egg/sperm bundle ascending speed following release; (D) Egg/sperm bundle dissociation time; (E) Percentage success following *in vitro* fertilisation. (mean ± SD)

	(A) Night	(B) Time of spawning after sunset (hrs)	(C) Bundle ascending speed (cm/sec)	(D) Bundle dissociation (mins)	(E) Fertilisation success (%)
A. tenuis	7	0.75-1 (19.20-19.30)	0.70 ± 0.15	23 ± 1.73	99 ± 1
A. millepora	9	2.5-3 (21.04-21.25)	0.72 ± 0.15	46.67 ± 4.16	98 ± 1.89
A. anthocercis	9	4.25-4.75 (22.49-23.20)	1.13 ± 0.25	137 ± 6.24	95 ± 4.58

Polar bodies were evident on multiple *A.tenuis* oocytes following dissociation (Fig 4.3 E, Fig 4.5 A&B), along with sperm cell aggregations adjacent to the recently detached polar bodies (Fig 4.5 C). During fertilisation, sperm cell aggregations were observed on the surface of oocytes (Fig 4.3 F&G, Fig 4.5 D), evident 15 mins following oocyte and sperm mixing (Fig 4.4 B) but increasing in number after 30 mins (Fig 4.4 C). However, despite fertilisation and first cleavage occurring at the animal pole (Martindale and Hejnol, 2009), sperm aggregations occurred over the whole oocyte surface and were not limited to this polar region (Fig 4.4 B).

Indeed, CLSM imaging of fluorescent marked histological sections indicated that sperm aggregations were not always located in close proximity of the oocyte nucleus (Fig 4.4 D). As previously noted (Okubo and Motokawa, 2007), cleavage occurs at the site of the oocyte nucleus. Early initiation of this was characterised by a central depression in which a sperm aggregation was evident, ringed with numerous other depressions as the oocyte ectoderm folds in during mitotic division (Fig 4.5 E & F). Sperm cells were still evident one hour post fertilisation within the cleavage furrow following first cell division (Fig 4.5 G & H).

Fertilisation rates were high (>95%) for all three species (Table 4.1 E, Fig 4.2 C), with no significant difference observed between species (Table 4.2 C). Stages of embryogenesis (Fig 4.3, Appendix 22, 23 & 24) across all three species were consistent with those previously reported for *Acropora in situ* (Okubo and Motokawa, 2007), with embryological developmental rates varying slightly, a result most likely due to temperature differences in this study to compared to Okubo & Motokawa (2007) (Fig 4.6).

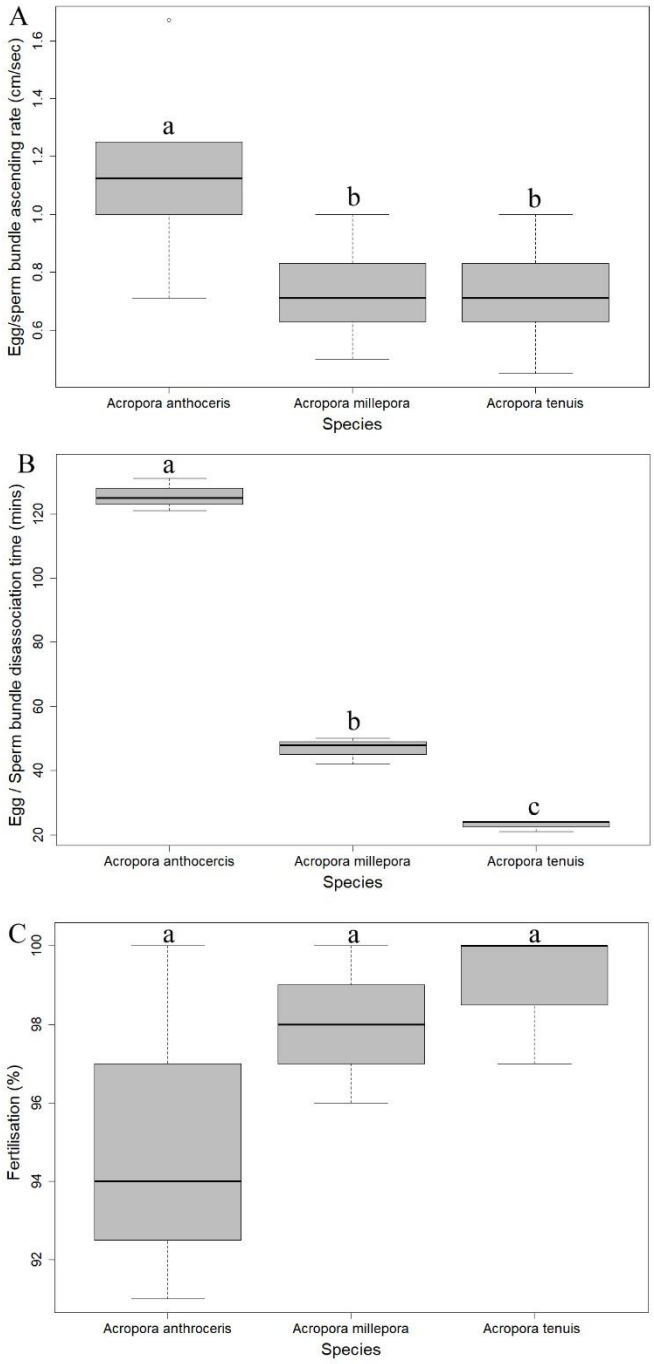


Figure 4. 2 Boxplots of *Acropora anthocercis*, *A. millepora and A. tenuis* egg/sperm ascending speed, bundle dissociation time and percentage fertilisation.

(A) egg/sperm bundle ascending speeds (cm/sec) following release *ex situ*; (B) bundle dissociation time (min); (C) Percentage fertilisation rate following in-vitro fertilisation. (mean ± se). The boxplots show the median (black line), the first and third quartiles (grey shaded box), and the lower and upper extremes, circles represent suspected outlying values. Different letters indicate significant differences between means (Linear regression, $p < 0.05$).

Table 4. 2 Results of species difference between *Acropora anthocercis*, *A. millepora* and *A. tenuis* on egg/sperm ascending speed, bundle dissociation time and percentage fertilisation.

(A) egg/sperm bundle ascending speeds following spawning; (B) egg/sperm bundle dissociation time; (C) percentage fertilisation success. Linear regression (LM).

A	*A tenuis*	*A millepora*	*A anthocercis*
A tenuis		p=0.683	p=<0.001
A millepora			p=<0.001
A anthocercis			
Overall model Adjusted R^2 = 0.510			

B	*A tenuis*	*A millepora*	*A anthocercis*
A tenuis		p=<0.001	p=<0.001
A millepora			p=<0.001
A anthocercis			
Overall model Adjusted R^2 = 0.993			

C	*A tenuis*	*A millepora*	*A anthocercis*
A tenuis		p=0.702	p=0.160
A millepora			p=0.274
A anthocercis			
Overall model Adjusted R^2 = 0.089			

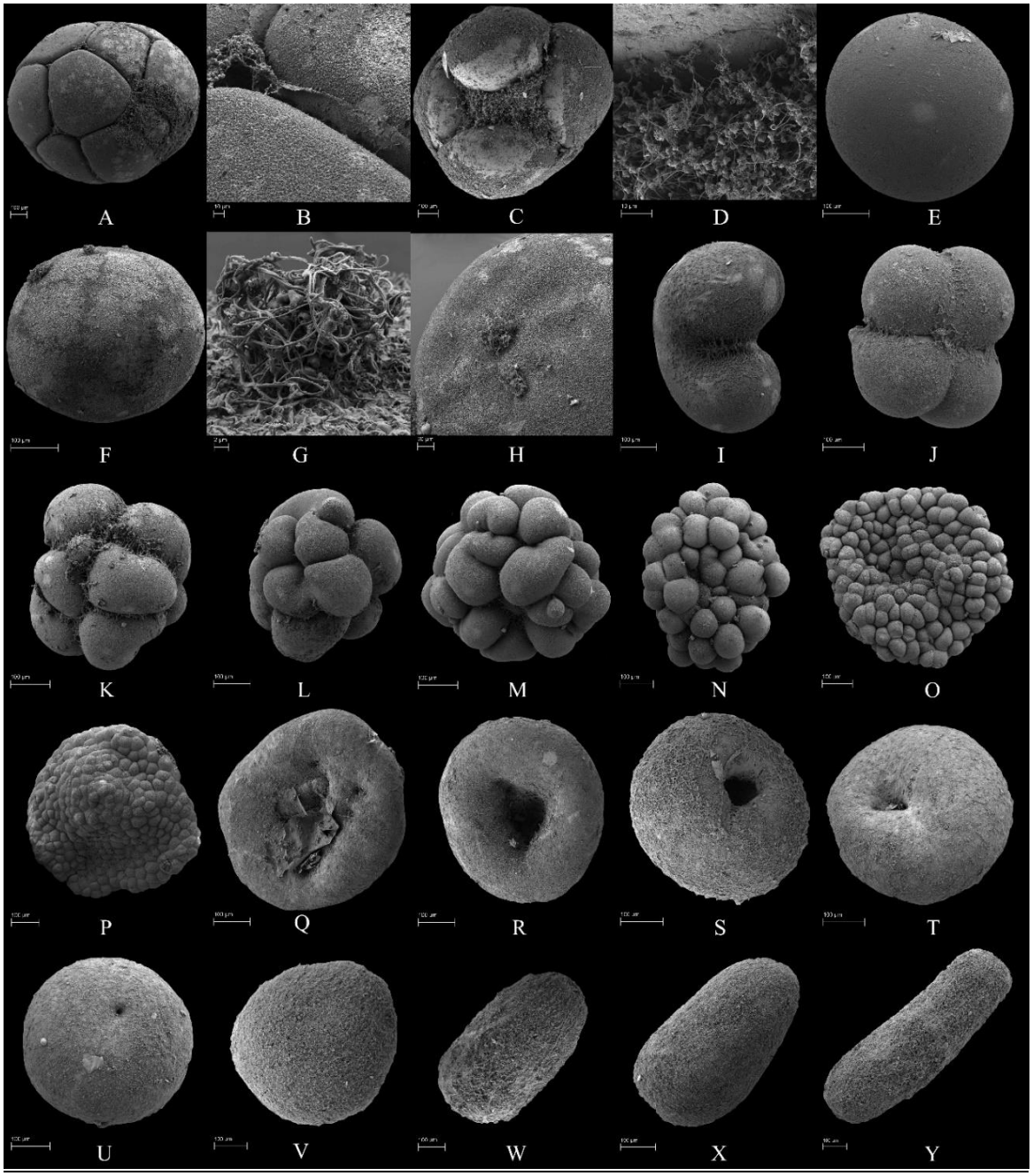


Figure 4. 3 SEM micrograph of a composite of three *Acropora* species embryogenesis (*A anthocercis*, *A. millepora* and *A. tenuis*).

(A) Newly released egg sperm bundle; (B) Sulphated mucosubstances holding egg/sperm bundle together; (C) Egg Sperm bundle dissociation; (D) Sperm liberation from the centre of the bundle during dissociation as sulphated mucosubstances break down; (E) Newly separated oocytes. Polar body visible adjacent to the animal pole; (F) Separate oocyte undergoing fertilisation; (G) Sperm penetrating oocyte's ectoderm; (H) Early initiation of first cleavage. Cleavage occurring at the site of the oocyte nucleus. Sperm cells visible in the centre of the

folding oocyte; (I) First cleavage / two-blastomere zygote (1-2 hrs post fertilisation hpf); (J) Four-blastomere stage (2-3 hpf); (K) Eight-blastomere stage (3-4 hpf); (L) 16-blastomere stage (3-6 hpf); (M-N) Morula (6-8 hpf); (O-P) Prawn chip (8-9 hpf); (Q-R) Bowl stage (10-17 hpf); (S-U) Gastrulation commences as the blastula rolls inward (17-28 hpf); (V) Tear drop stage (54-66 hpf); (W-Y) Planula elongation stage (78-102 hpf). Scale = 2µm (G), 10µm (B&D), 20µm (H), 100µm (A,C,E-F,I-Y). *All images taken by J Craggs*

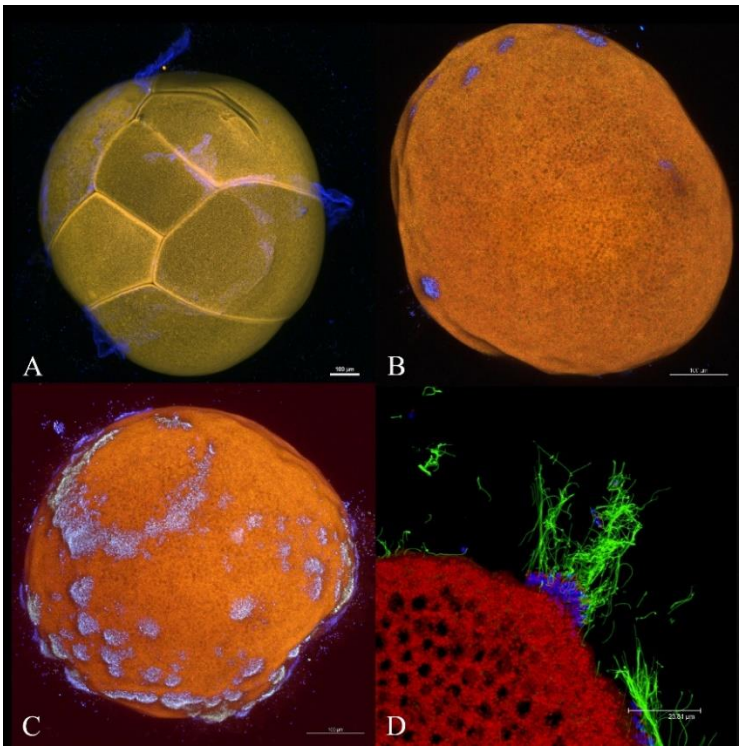


Figure 4. 4 Confocal laser scanning photomicrograph of *Acropora millepora* gametes.

(A) Newly released egg sperm bundle, with sperm (blue) release from the bundle; (B) Initial sperm aggregations on oocyte ectoderm 15 mins after oocyte/sperm mixing; (C) Sperm aggregations on oocyte's ectoderm 30 mins after oocyte/sperm mixing; (D) Histological section of oocyte with sperm aggregation on the surface 30 mins after oocyte/sperm mixing. Sperm nuclei (Panel A,B,C,D: blue) stained with DAPI. Sperm microtubules labelled with Alpha Tubulin Mouse Monoclonal Antibody at 2 µg/mL in 0.1% BSA and incubated for 3 hrs at room temperature and then labelled with Goat anti-Mouse IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 488 conjugate a dilution of 1:2000 for 45 mins at room temperature (Panel D: green). Actin cytoskeleton stained with Alexa Fluor® 555 Rhodamine Phalloidin (Panel D: red). Scale = 100 µm A,B &C), 23.81 µm (D). *Images A-C taken by J Craggs*. *Photograph credit; D* © Dr David Robertson, The Institute of Cancer Research.

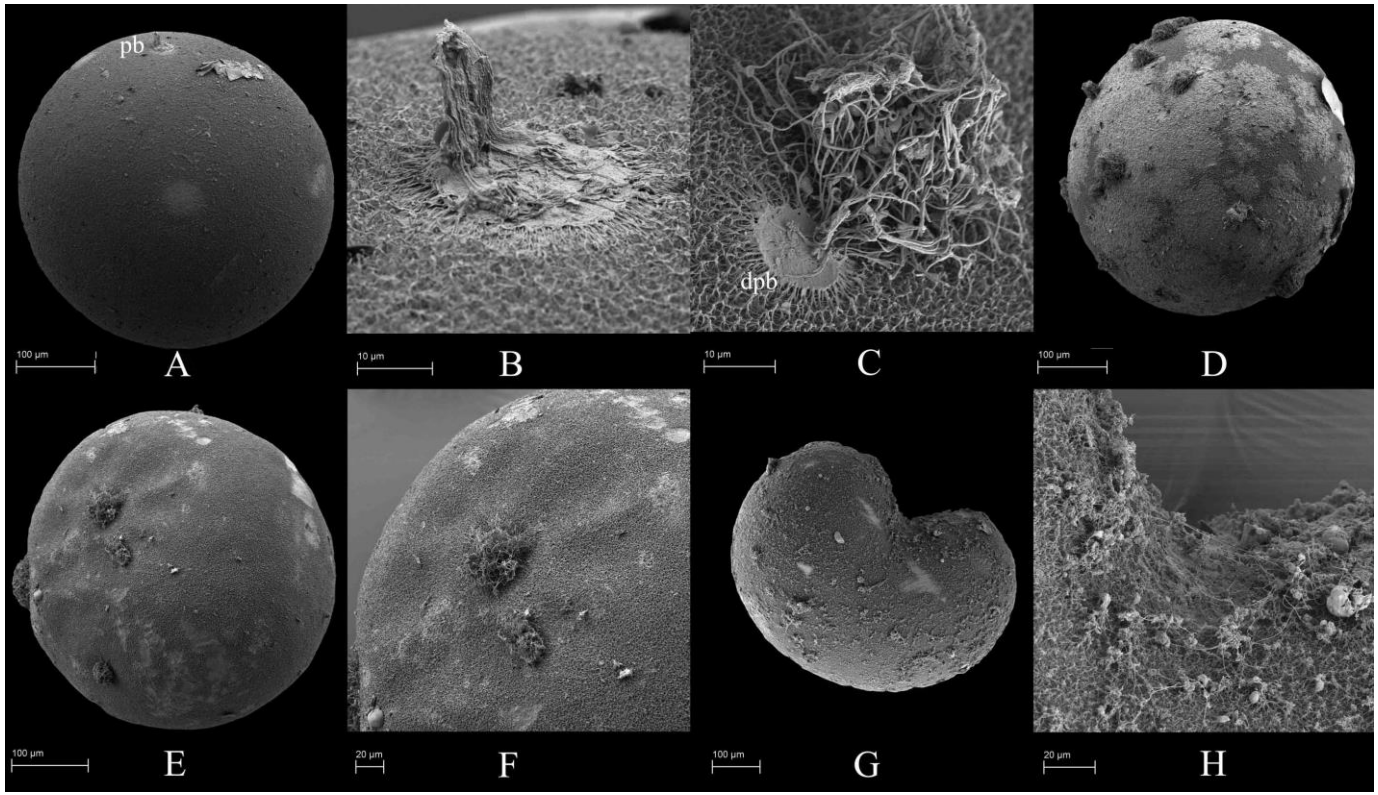


Figure 4. 5 SEM photomicrograph of *Acropora tenuis* embryos.

(A) Newly separated oocytes with polar body (pb); (B) Enlargement of Figure 5 A showing the pb; (C) Sperm aggregations adjacent to site of detached polar body (dpb); (D) Sperm aggregations on the oocyte surface 45 mins following sperm/oocyte introduction; (E) Early initiation of first cleavage; Sperm aggregation at the oocyte cell division site, ringed by depressions of the oocyte ectoderm; (F) Enlargement of Figure 5 E showing the sperm aggregations; (G) First cell division and formation of two cell blastomere; (H) Sperm cells in the cleavage furrow of a two cell blastomere. Scale = 10µm (B & C), 20µm (F & H), 100µm (A,D,E & G). *All images taken by J Craggs*

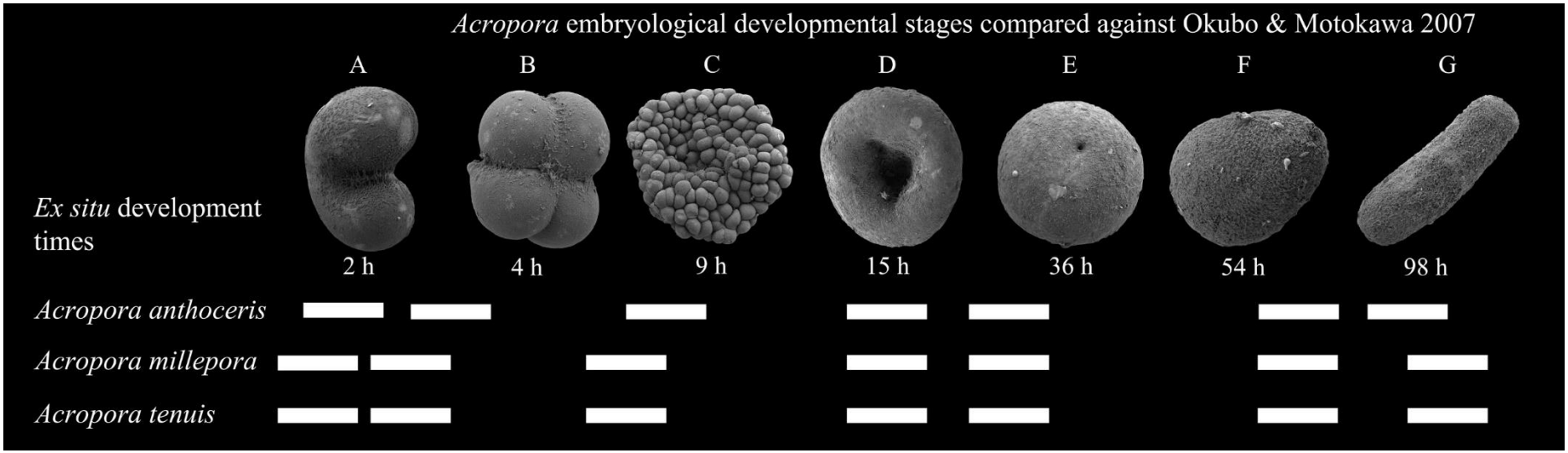


Figure 4. 6 Comparison of *Acropora* embryological development times *ex situ* vs *in situ*.

(A) first cleavage; (B) four-cell stage; (C) prawn chip stage; (D) bowl stage; (E) round stage; (F) tear drop stage; elongated stage. *In situ* definitions from Okubo & Motokawa 2007. *All images taken by J Craggs*

4.6 Discussion

The majority of studies focusing on sexual reproduction of broadcast corals, particularly for early embryological developmental stages have been conducted in tropical institutions that are close to natal reefs (Marlow and Martindale, 2007; Chui *et al.*, 2014; Guest *et al.*, 2014). This was because the spawning of corals in closed systems *ex situ* (Craggs et al. 2017, Chapter 3) only recently became possible. In this Chapter, I aimed to investigate whether corals that have developed gametes, and subsequently spawned *ex situ* (Chapter 3) show normal embryological development in line with those previously described with coral spawning *in situ*. All cell stages (ranging from gametes to fully formed planula larvae), were preserved from two species, *Acropora tenuis* and *Acropora millepora* that completed full gametogenic cycles *ex situ*, and for a third species, *Acropora anthocercis*, in which colonies were imported one month prior to the predicted spawning date.

Gametes developed within broodstock coral colonies housed in open seawater tanks (aquariums), which are exposed to natural photoperiod and lunar cycles can be smaller in size (than their wild counterparts), a result which has been highlighted as inferring inferior quality to those produced in natural settings (Lin *et al.*, 2018). However, this is quite difficult to ascertain as newly released *Acropora* oocytes in corals *in situ* can show considerable variation in size (402.5 - 652µm mean diameter, here representing a range of sizes from 22 species, including *A. millepora* and *A. tenuis*) (Wallace, 1985; Arai *et al.*, 1993; Mangubhai and Harrison, 2006; Okubo and Motokawa, 2007). That said, in this study the oocytes of *A. millepora* and *A. tenuis* oocytes (developed *ex situ*) were slightly smaller (385 ± 36 µm & 396 ± 22 µm, mean ± SD respectively) (Appendix 22 & 23). That is compared to the smallest recorded range for *in situ* Acroporids (identified above). However, the oocytes released from the newly imported *A. anthocercis* colonies (472 ± 28µm, mean ± SD) (Appendix 24) were within this range, although towards the lower end. Therefore it remains unknown if this apparent reduction in oocyte size was due to *ex situ* development or because of the natural variations in oocyte diameters across species of acroporids. Broodstock nutrition (as a confounding factor of a captive environment as discussed in Chapter 3) may be a contributing factor here, as it has been shown to influence gamete quality in maricultured marine invertebrates (Berntsson *et al.*, 1997; Carboni *et al.*, 2015). Indeed, studies on temperate sea urchins have shown formulated diets (high-protein diets with cholesterol and β-carotene) can

positively influence egg energy and egg size and this resulted in enhanced survival in recruiting juvenile forms (De Jong-Westman *et al.*, 1995). However, positive transfer of survival gains from increased gamete quality to larva is not ubiquitous within all marine invertebrates (Calado *et al.*, 2010; Carboni *et al.*, 2015) and is therefore not a complete indicator of fitness.

Fertilisation rates within all three species from this study were high (>95%), which is consistent with those previously reported, both *in situ* (Miller and Mundy, 2005) and from experiments in which gametes were collected from broodstock colonies and transported to flow through aquariums a few days prior to spawning (Negri, Marshall and Heyward, 2007; Humphrey *et al.*, 2008; van Oppen *et al.*, 2014). In addition, no embryological development abnormalities were observed in any of the three species, suggesting that no lasting adverse effects occur with *ex situ* produced corals.

Another benefit of exploring the early life stages of corals bred *ex situ* (as was undertaken in the Chapter) is the ability to understand more about the fertilisation process and the structural formation of the gametes and how these develop anatomical features in the adult form. It has already been shown that apical-basal polarity of cnidarian oocytes forms in the germinal epithelium within the adult (Eckelbarger, Hand and Uhlinger, 2008). Following oocyte meiotic reduction divisions' polar bodies are formed, defining the animal polar region, and eventually the orientation of the mouth in the adult form (Momose and Schmid, 2006). The site of the polar bodies corresponds to the unipolar cleavage furrow following fertilisation (Martindale and Hejnol, 2009), and is therefore the suspected sperm entry site into the oocyte for successful fertilisation. Indeed, such polar bodies have been observed on the surface of many scleractinian oocytes (Babcock and Heyward, 1986; Okubo and Motokawa, 2007; Okubo *et al.*, 2013) and sperm aggregations have been show on the surface of the oocytes during fertilisation of *Platygyra sinensis, Goniastrea aspera* and *Montipora digitata* (Babcock and Heyward, 1986). Through the application of CLSM and SEM I was able to show that sperm aggregation in two *Acropora* species also occurs (*A.millepora* Fig 4.4C & *A.tenuis* Fig 4.5D), but they show no polarity (i.e. not limited to the animal pole). However, despite the lack of polarity, sperm aggregations do appear to occur adjacent to the site of recently detached polar bodies (Fig 4.5C). Further, it appears only sperm within these aggregations in the animal pole region, as seen in sperm clusters in the centre of surface folds on the oocyte during early initialisation of the cell division and therefore nucleus location (Fig 4.5 E&F), that were responsible for successful fertilisation.

Spawning times of *A. tenuis* and *A. millepora* were consistent with those recorded *in situ* (Babcock *et al.*, 1986). This supports previous work in that the closed mesocosms were able to replicate the environmental conditions enabling accurate induction of spawning at a given time (Craggs et al. 2017, Chapter 3). Interestingly, Babcock et al. (1994) recorded *A.anthoceris* spawning on the west coast of Australia at 22.50. *Ex situ* observations in this study suggest that spawning for this species will occur on the GBR within the same timeframe 22.49-23.20, indicating that across the continent *A. anthocercis* spawns later than *A. tenuis* and *A. millepora,* and is consistent regardless of geographical area.

There were significant differences in the egg/sperm bundle ascending speeds and dissociation times between the three species, which was unexpected. This provides further insight into reproductive isolation barriers and driving mechanisms for long term evolutionary divergence. For example, during multispecific synchronous spawning events, gamete mixing would provide an opportunity for interspecific hybridisation to occur (Miller and van Oppen, 2003). Indeed Willis et al (2006) indicated that interspecific crosses could provide relatively high fertilisation success (>45%) and identified this as an important evolutionary capability of broadcast spawning corals enabling rapid speciation. However, despite the possibility of creating crosses *ex situ*, it is thought that such interspecific crosses may be rare *in situ* as a result of pre-zygotic barriers, such as fine-scale temporal patterns of spawning behaviour and limited fertilisation compatibility between species (Wei *et al.*, 2012). Varying dissociation times may be another method utilised to reduce such crosses as well. Such variation has also been shown to occur within taxonomically similar species of coral such as the *Acropora humilis* species group (Wolstenholme 2004). It is thought that even though the *A.humilis* species group can probably cross and successfully produce viable offspring, the variation in dissociation again reduces the chances of this occurring.

In addition to dissociation differences, significant variation in the egg/sperm bundle ascending speeds were observed. This may be associated with species environmental spatial zonation. Bundle buoyancy is a result of wax ester concentrations within the oocytes (Padilla-Gamiño *et al.*, 2013), and differences in wax esters have been shown to occur between species with *A. millepora* and *A.tenuis* having overall lipid contents of 69.1 and 62.5% respectively (Arai *et al.*, 1993). Although the lipid content of *A. anthocercis* oocytes is not currently known, it may be predicted that it is much greater than that of both *A. millepora* and *A. tenuis* due to the ascending speeds for this species being significantly greater than for the other two. *A.anthocercis* may require a faster ascending time as these corals typically inhabit shallow,

high wave energy exposed reefs compared to the more sheltered sites from which *A. tenuis* colonies were collected. Such an adaptation may minimise physical damage to the bundles resulting from hitting the reefs within turbulent waters. Furthermore, a protracted dissociation time might facilitate movement (by currents) of the gametes to calmer waters, in turn minimising the potential confounding effects of sperm dilution in more agitated waters. However, it is unknown if bundle ascending rates vary within the same species across different habitats, e.g. shallow, sheltered reef flats and lagoons, more exposed reef crests and the deeper mesophotic reefs. As the practice of assisted gene flow is being explored (van Oppen *et al.*, 2014, 2017) i.e. the movement of corals from one place to another, understanding this aspect of a coral's lifestyle may prove to be important to ensure transplanted corals reproduce successfully in their new environment, as well as having the potential to spread to new areas.

In conclusion this Chapter illustrates that spawning and embryological development are consistent in *ex situ* reared corals compared to their wild counterparts. Although oocytes of *ex situ* reared corals were slightly smaller than in the wild, there was no detectable abnormal developments, and fertilisation success remained high. Taken together, these indicate that studies utilising *ex situ* reared coral embryos, larvae, recruits and adults will be comparable to any conducted from wild stock collected directly from reefs.

Chapter 5: Interspecific hybridisation and multi genotype aggregations enhance survivorship during early ontogeny in a reef building coral

5.1 Abstract

Complex interactions influence post settlement coral survival and understanding these processes are critical in order to overcome high mortality rates if reef restoration practises are to be up-scaled. Broadcast spawning is often synchronised across coral species leading to interspecific hybridization occasional occuring during such spawning events. Following a relatively short embryological pelagic phase, developed larvae either settle as individual primary polyps or aggregate in multi-genotype entities. Hybridisation vigour and the influence of these multi–genotype entities have both (independently) been shown to improve post settlement survival. However, no studies have explored both effects together. In this Chapter, I aimed to assess survivorship and growth of early post-settlement coral spat of two pure crosses (*Acropora millepora* and *A. anthocercis*) and an interspecific hybrid (*A. millepora*/*A. anthocercis*). In addition, the effect of genotype numbers within an entity on survivorship over a 12 week period and growth between week two to six was assessed. Survivorship was highest in the interspecific hybrid, and significantly greater than the pure cross of *A. millepora* but not that of *A.anthocercis*. This suggests hybridisation vigour was not ubiquitous. Growth in the interspecific hybrid was also highest but this was only significant compared to the pure cross of *A.anthocersis.* Further, post settlement entities with two or more genotypes, exhibited enhanced survivorship. Peaks in survivorship occurred in the pure genetic crosses with (approximately) three genotypes, but this peak shifts to greater genotype numbers in the interspecific hybrid entities. Such size-mediated responses therefore positively influence survival in all genetic crosses and were consistent with previous studies. The post settlement benthic environment is highly stochastic, with factors not included in this study affecting mortality rates. Research into methods that control these negative interactions may improve the productivity of sexually produced coral spat for research and reef restoration practices and warrant further investigation.

5.2 Introduction

Reef building corals typically exhibit a Type III survivorship curve characterised by the greatest mortality (lowest age-specific survival) early in life, with relatively low rates of death (high probability of survival) for those surviving this bottleneck (Wilson and Harrison 2005; Edward S. and Deevey 1947). This is driven by complex relationships associated with settlement orientation and light (Babcock and Mundy, 1996), and sedimentation (Maida, Coll and Sammarco, 1994), which all influence early life mortality. In addition competitive benthic interactions, occurring at small spatial scales have also been documented for this life stage in corals (Vermeij 2006). For example, sponges (Aerts and Van Soest, 1997), macroalgae (Harriott, 1983; Kuffner *et al.*, 2006), bryozoans (Dunstan and Johnson, 1998) and crustose coralline algae (Harrington, 2004) have all been shown to be capable of outcompeting coral spat.

For most organisms, the ability to grow fast can strongly influence survivorship. This is true of corals, with reduced mortality being directly correlated to increasing colony size (Vermeij, 2006; Guest *et al.*, 2014). This has in turn been linked to improved resource acquisition and energy partitioning (Raymundo, 2004). Up to 80 % of a hermatypic coral's carbon budget requirement (for respiration) is assimilated through the translocation of photosynthetically fixed carbon from Symbiodiniaceae (Muscatine, McCloskey and Marian, 1981; Tremblay *et al.*, 2012). The remainder of this budget requirement is gained through heterotrophic input i.e. prey capture (Houlbrèque and Ferrier-Pagès, 2009). During early ontogeny, some corals lack their Symbiodiniaceae and so the ability to efficiently feed (heterotrophically) can enhance spat survivorship (Conlan *et al.*, 2017) and size mediated improvement of capture efficiency is an important factor in this.

Following pelagic embryogenesis, the larvae of broadcast spawning corals metamorphose forming a 'primary' polyp. Settlement (when it occurs) can be in the form of a single genotype or multi-genotype entities, the latter being a result of the larvae's propensity to aggregate (Puill-Stephan *et al.*, 2012a). Single genotype entities appear to be competitively inferior, and show lower survivorship rates compared to multi-genotype entities (>2 genotypes), a result likely due to the larger size of the multi-genotypes, particularly in the early, and most sensitive phases of ontogeny. However, those single genotypes that do survive show faster growth rates at the genotype level than the multi-genotype entities (Amar, Chadwick and Rinkevich, 2008). These findings suggest enhanced species survival strategy of multi-genotype entities at the population

level, i.e improved initial survival of co-inhabiting genotypes within a multi-genotype entity, but that individual genotypes within those entities have trade-offs in growth.

During synchronised multi-specific spawning events, interspecific hybrids of reef building coral can form (Willis *et al.*, 1997; Miller and van Oppen, 2003). Whilst thought to be relatively rare (due to low chances of successful fertilisation) (Willis *et al.*, 1997), hybridisation, particularly within the genus *Acropora*, has likely been a major influence in the diversification of this genera over large scale geological time periods (Willis *et al.*, 2006). Indeed, it has been hypothesised, that reticulated evolutionary pathways also play an important role in species range expansion and adaptation to changing environments (Willis *et al.*, 2006). For example, compatible hybrid crosses have recently been shown to exhibit increased thermal tolerance and resilience at elevated pCO_2 (Chan *et al.*, 2018).

In this study, I quantitatively assessed the effect genotype numbers within newly settled entities had on the survivorship and growth during early ontogeny of two acroporid species from the Indo Pacific, *Acropora millepora* and *Acropora anthocercis*. Interspecific hybrid corals, created by crossing *A. millepora* oocytes and *A.anthocercis* sperm were also assessed with regard to evidence of hybridisation vigour enhancing survivorship and growth compared to pure crosses of genotype aggregations.

5.3 Methods

5.3.1 Ex situ spawning, gamete collection and fertilisation

Six gravid colonies (<20 cm^2) of *Acropora millepora* and *Acropora anthocercis* were transported from the Great Barrier Reef, Australia to London in September 2016 (CITES import permit number: 545320/03) (Craggs *et al*., 2018, Chapter 2). The colonies (or broodstock) were placed in a purpose built closed system mesocosm, designed to replicate the conditions required to induce spawning *ex situ* including seasonal temperature change, photoperiod, solar irradiance and lunar cycles (Craggs *et al*., 2017, Chapter 3). The spawning date for the broodstock was predicted based on *in situ* species' spawning patterns (Babcock *et al.*, 1986) and from direct observation of oocyte pigmentation development within fragments removed from each colony two to three days prior to artificial full moons (16th October and 14th November 2016). Thirty minutes prior to the predicted spawning date and time, water

movement within the mesocosms was turned off and one gamete collector per broodstock genotype was positioned over the colonies. These collectors were held in place using attachment clips to maintain their correct location. Using torches with red light, colonies were checked every 15 mins for signs of bundle setting (Edwards *et al.*, 2010). Four colonies of *A. millepora* and four colonies of *A. anthocercis* spawned between 21:00 to 23:00 on 23rd November 2016, nine NAFM. Released bundles were surface skimmed from within the gamete collector with a beaker, and gently hand stirred for 30 to 45 mins to aid the dissociation of sperm and oocytes. The sperm was then siphoned from the beaker, density estimated visually based on water opacity, and an optimal density (10^{-5} - 10^{-6} sperm cells / ml) acquired to ensure highest fertilisation success (Oliver and Babcock, 1992). The oocytes were rinsed three times with newly mixed sea water (NMSW).

Three 'treatments' were generated (by mixing oocytes and sperm from the appropriate gamete isolations). The genetic combinations included: two pure species crosses, *A. millepora* / *A. millepora* and *A. anthocercis* / *A. anthocercis* and one interspecies hybrid *A. millepora* / *A. anthocercis* (here after represented as AM/AM, AA/AA and AM/AA respectively). In the representation of these crosses, oocyte donor species is followed by sperm donor species. A second interspecific hybrid (*A. anthocercis* / *A. millepora* AA/AM) was attempted but resulted in very low fertilisation successand so cultures were discarded.

Oocyte/sperm mixes were stirred every 5 mins for 45 mins to allow fertilisation to take place (Edwards *et al.*, 2010). Fertilisation was 'ended' by siphoning the sperm off and rinsing the eggs in NMSW. Zygotes were then transferred to 6 litre culture bowls (two bowls for each genetic cross) and placed in a water bath at the same temperature as the broodstock colonies ($27.7 \pm 0.1C^{o}$). After two to three hours three aliquots containing more than 50 zygotes from each cross were taken, photographed and fertilisation success rates calculated using ImageJ (Table 5.1).

Developing embryos are susceptible to fragmentation during the early stages of embryogenesis, due to the shearing forces from water movement (Heyward and Negri, 2012). Static culture bowls, therefore, received no water changes during the first 24 hrs until the embryos had developed past the fragile period and into the late 'prawn chip' stage (Okubo and Motokawa, 2007). After this, an 80% water change was completed daily, by gravity siphoning water from the base of the bowl, leaving the lipid rich embryos floating at the surface. Following water changes, cultures were refilled with aquarium system water. Embryogenesis took four days,

after which free swimming planula from each treatment were transferred into six separate 6 litre aquariums, two for each treatment. Each tank contained 45 preconditioned coral settlement plugs (Ocean Wonders) covered in a biofilm to facilitate larvae settlement (Morse *et al.*, 1996; Webster *et al.*, 2004).

5.3.2 Production of entity classes

The settlement tanks were placed in a temperature control bath at 27.7 ^{o}C for one week to allow settlement, receiving 80% water changes daily to maintain water chemistry parameters. Larvae either settled as individual genotypes or formed entities with more than two aggregated genotypes (Goreau, Goreau and Hayes, 1981; Amar, Chadwick and Rinkevich, 2008). In order to investigate the role that genotype number (within settled entities) has on survivorship and [genotype] growth rate four entity treatments were studied, (1 genotype, 2 genotype, 3 genotype and >3 genotype entities) and settlement plugs were divided in order to maximise the number of each entity type for each treatment (Table 5.2). Plugs were glued to a 20 x 20 cm UPVC sheet in a grid formation, photographed and, using Photoshop, a red mark was placed to the left of the entity to produce a map for each treatment, aiding rapid location of the entity being tracked for subsequent imaging (Appendix 25). Each UPVC sheet was replicated three times for each treatment and coded, AM/AM (1-3) (Appendix 26), AA/AA (1-3) (Appendix 27), and AM/AA (1-3) (Appendix 28). Treatment replicates were placed in three separate tanks supplied with water from a centralised mesocosm. A second map for each treatment was produced to identify the number of genotypes within each entity class. Genotype numbers were annotated in Photoshop (Fig 5.1) and provided the starting entity types and genotype numbers for each genetic cross (Table 5.2).

5.3.3 Measuring entity and genotype surface area and survivorship

Using these maps all entities were photographed on week two, four, six, eight and 12 using a Canon 5D MkIII with MPE65mm fixed focal lens, at x5 magnification. As the entities grew magnifications were reduced and corresponding scale images taken. Corresponding image numbers from each entity were used in ImageJ to measure genotype survivorship and entity surface area. Genotype linear growth within each entity type and genetic cross was calculated approximating each genotype to a circular form and an average growth of the radius between week two and week six was estimated using the following equations:

$$\sqrt[2]{\frac{A_T}{\pi}} = R_T$$

Where $A_{T,}$ is the genotype area at time $_T$, and R_T is the genotype radius at time $_T$.
Average radius increase between week two to week six was calculated as follows:

$$\frac{R'_{T,2} + R'_{T,6}}{2} = M_{2-6}$$

And the final linear radial growth (change) between each time point was calculated by dividing the difference between the radius between week six and week two divided by the average radius increase:

$$\frac{R'_{T,6} - R'_{T,2}}{M_{2-6}} = r$$

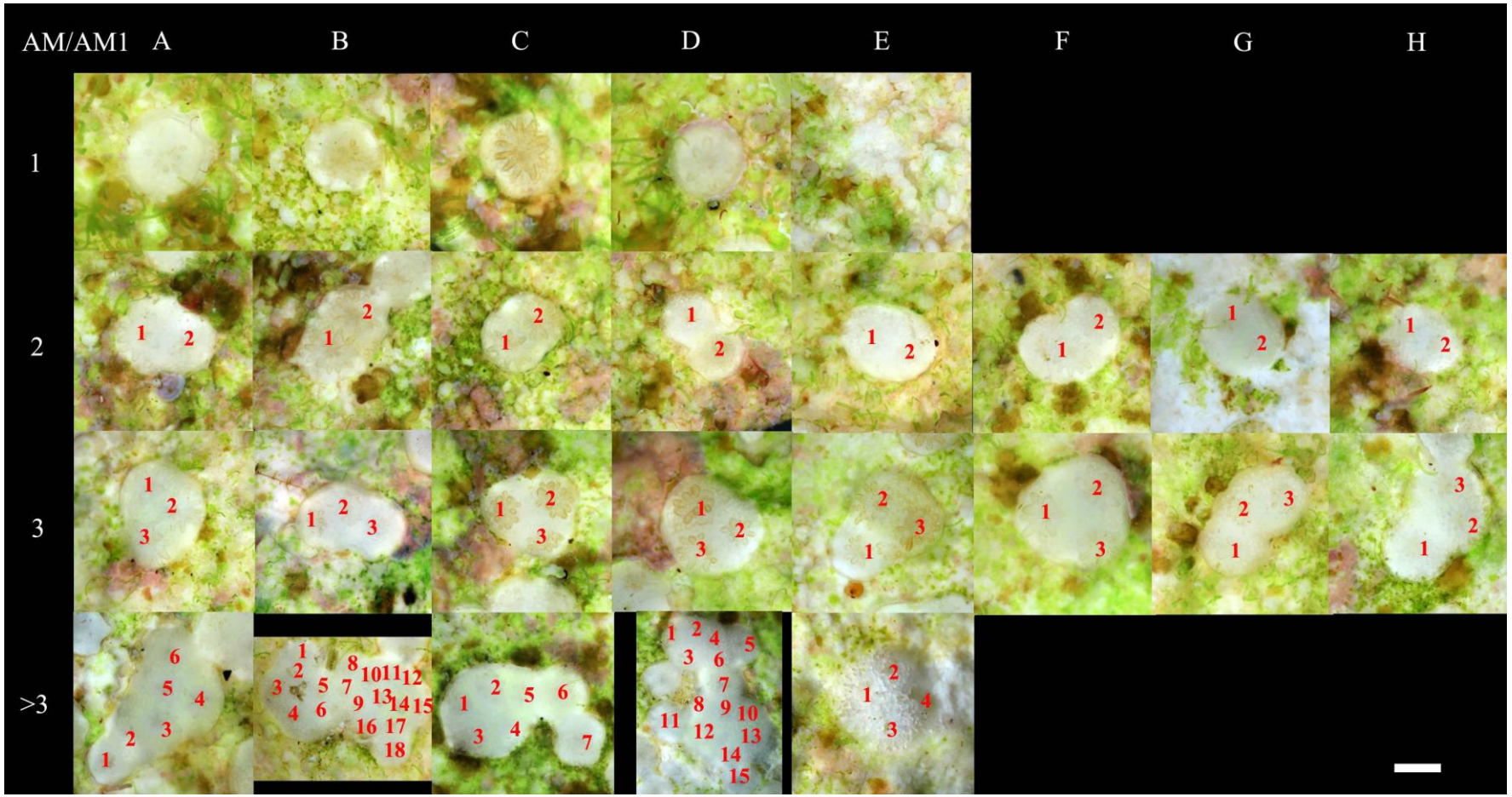


Figure 5. 1 Genotype maps of entity types and genetic cross.

Genotype numbers within the entity types are shown on the y axis (1 genotype, 2 genotype, 3 genotype and >3 genotype entities) and expressed numerically over the settled coral colony. Entity type replication within treatment is on the x axis (A-H). Treatment *Acropora millepora* / *A. millepora* (AM/AM) replicate 1 represented. Scale = 1 mm. *All images taken by J Craggs*

Table 5. 1 Comparison of percentage fertilisation (% mean ± SD) in pure crosses of *Acropora anthocercis/A. anthocercis* (AA/AA), *Acropora millepora/A. millepora* (AM/AM) and interspecific hybrid crosses *Acropora millepora/A. anthocercis* (AM/AA) *A. anthocercis*/*A. millepora* (AA/AM).

Genetic cross Mother / father	Fertilisation success (%)
AA/AA	91.12 ± 4.80
AM/AM	98.08 ± 1.89
AM/AA	65.62 ± 5.42
AA/AM	0.37 ± 0.64

Table 5. 2 Total numbers of each entity type (1 genotype, 2 genotype, 3 genotype and >3 genotype entities), total numbers of genotypes expressed within entity types (n), across three replicates of the three treatments (AA/AA, AM/AM and AM/AA)

Mother / father	1 genotype entity type (Nº genotypes)	2 genotype entity type (Nº genotypes)	3 genotype entity type (Nº genotypes)	>3 genotype entity type (Nº genotypes)	Total entities/ genetic cross (Nº genotypes)
AA/AA	15	21	6	3	45
	(15)	(42)	(18)	(21)	(96)
AM/AM	15	24	24	15	78
	(15)	(48)	(72)	(170)	(305)
AM/AA	15	18	18	18	69
	(15)	(36)	(54)	(67)	(172)

5.4 Statistical analysis

Coral survivorship at 12 weeks and linear genotype coral growth between two to six weeks, both within and between the three genetic crosses (AM/AM, AA/AA & AM/AA) and across the different entity types (1, 2, 3 & >3 genotypes) was analysed using general linear models. Model assumptions of normality and homogeneity were tested using standard residual diagnostics and overly influential data points were tested for, using Cook's distances following Zuur et al (2007). Model assumptions held and there were no overly influential data points.

The analyses were conducted with the nlme packages in R: Statistical Computing Software (R Development Core Team, 2015).

5.5 Results

Survivorship of post-settled coral spat over 12 weeks was influenced by the genetic cross (pure *Acropora millepora* (AM/AM), *Acropora anthocercis* (AA/AA) and the interspecific hybrid cross of *A. millepora* oocytes and *A. anthocercis* sperm (AM/AA), together with the number of genotypes within a settled entity (Entity types: 1 genotype, 2 genotypes, 3 genotypes and >3 genotypes).

Survivorship was highest in the interspecific hybrid cross AM/AA (50%, Table 5.4). However, there was no significant difference in surviving entities between AM/AA and AA/AA ($p = 0.348$) (Table 5.3A, Fig 5.2 A). AM/AM showed lowest survivorship overall (14.1%, Table 5.4) and this was significantly different from the other two crosses ($p<0.001$).

With genetic crosses pooled, entities with 2, 3 and >3 genotypes all had significantly higher survivorship than single genotype entities ($p=0.003$, $p<0.001$ and $p=0.031$ respectively) (Fig 5.2 B & Table 5.3 B). A settled entity with 2 or 3 genotypes appeared optimal (with regard to survivorship) compared to both 1 and >3 (Table 5.3 B, Fig 5.2 B). Additionally, a bimodal pattern in survivorship within the >3 entities occurred, with two points of increasing mortality occurring after 4 and 12 weeks (Fig 5.2 C).

When each genetic cross was analysed separately, the same pattern was also observed in the pure crosses AM/AM & AA/AA, with 2 or 3 genotypes showing highest overall survivorship (Fig 5.2 D). Entities with 3 genotypes in the AA/AA cross showed an increase in survivorship compared with those of 2 genotypes (Fig 5.2 D), but this difference was not statistically different ($p = 0.366$) (Table 5.3 D). A different pattern was evident for the interspecific hybrid AM/AA, with increasing survivorship from 1 genotype and peaking at >3 genotype entities, with >3 genotypes exhibiting the highest rates of survivorship across all entity types and across the three genetic crosses (Fig 5.2 D).

It should be noted that an effect of tank on survivorship was detected, with corals in tank 2 having a significantly lower survivorship than those in tanks 1 and 3 ($p<0.001$) (Table 5.3 D,

Fig 5.2 E). However, when the data was analysed with this in mind there was no change in the survivorship trends (Fig 5.2 F).

There was no tank effect on growth (Fig 5.3 A) and there was also little difference in genotype linear growth from week 2 to 6 between the genetic crosses. Indeed, there was no significant difference in growth rate between AM/AA and AM/AM or AA/AA and AM/AM (p=0.476 and p=0.131 respectively). However, there was a significant difference between AA/AA and AM/AA (p=0.030), with the latter hybrid cross growing slightly faster (Table 5.5 B).

Similar, to the effects on survivorship, there were effects on genotype linear growth rate between week two and six depending on the genotype numbers within entity. Entities with 2,3 and >3 genotypes all had significantly higher growth than those with only 1 (p<0.001, p<0.001 and p<0.001 respectfully) (Fig 5.3 C). However, in contrast to that for survivorship, there was no significant difference in growth rate between 2, 3 and >3 genotype entities (p=0.689, p=0.375 and p=0.573 respectively, Table 5.5 C). However, when the distinct crosses were analysed separately, the pattern only held true for the pure cross (AM/AM) (Table 5.5 D, Fig 5.3 D). For the pure cross AA/AA and the interspecific cross AM/AA showed no significant difference in growth between any entity types (Table 5.5 D, Fig 5.3 D)

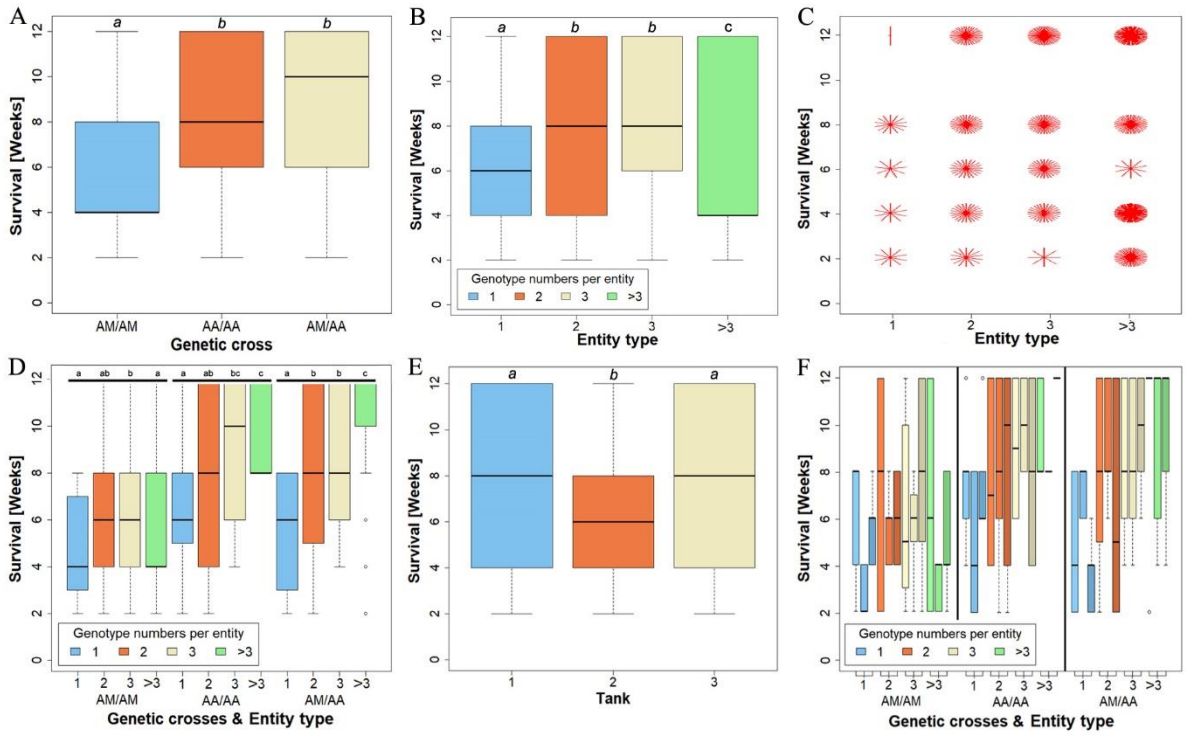


Figure 5. 2 Coral spat survivorship over 12 weeks within three genetic crosses and different entity types.

Pure *Acropora millepora* cross *(*AM/AM), pure *Acropora anthocercis* cross (AA/AA), interspecific hybrid *A. millepora/A. anthocercis* (AM/AA), represented as dam / sire. Entity types - 1 genotype, 2 genotypes, 3 genotypes and >3 genotypes. (A) Survivorship of three genetic crosses with entity types pooled; (B) Survivorship of entity type with three genetic crosses pooled; (C) Survivorship sunflower plot of entity type with three genetic crosses pooled; (D) Survivorship of coral at entity and genetic cross level; (E) Influence of tank on overall coral survivorship with genetic crosses and entity types pooled; (F) Survivorship of coral with genetic crosses, entity types and tank replicates separated (Light coloration = tank 1, medium colouration = tank2 and dark colouration = tank 3). Boxplots show the median, the first and third quartiles, and the lower and upper extremes. Different letters indicate significant differences between means (Linear regression, $p < 0.05$).

Table 5. 3 Results of linear regressions of pure genetic crosses of *Acropora millepora* (AM/AM) and *Acropora anthocercis* (AA/AA) and interspecific hybrid *A. millepora*/*A. anthocercis* (AM/AA), represented as dam / sire, on spat survivorship with varying genotype entities (Entity type - 1 genotype, 2 genotypes, 3 genotypes and >3 genotypes).

(A) Overall survivorship between genetic crosses; (B) Influence on tank effect on survival; (C) Survivorship of genotypes within entity types with all genetic crosses pooled; (D) Survivorship of genotypes within entity types with genetic crosses separated.

A	AA/AA	AM/AM	AM/AA
AA/AA		*p*<0.001	*p*=0.348
AM/AM			*p*<0.001
AM/AA			
Overall model p< 0.0001 & Adjusted R^2 = 0.177			

B	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p*=0.003	*p*<0.001	*p*=0.031
2 genotype			*p*= 0.245	*p*=0.137
3 genotype				*p*=0.003
>3 genotype				
Overall model p=0.0005014 & Adjusted R^2 = 0.026				

C – AM/AM	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p*=0.111	*p*=0.020	*p*=0.7275
2 genotype			*p*=0.309	*p*=0.022
3 genotype				*p*<0.001
>3 genotype				
Overall model *p*= 0.0003134 Adjusted R^2 = 0.051				
AA/AA	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p*=0.113	*p*=0.038	*p*=0.003
2 genotype			*p*=0.366	*p*=0.046
3 genotype				*p*=0.376
>3 genotype				
Overall model *p*=0.02272 & Adjusted R^2 = 0.069				
AM/AA	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p*<0.001	*p*<0.001	*p*<0.001

2 genotype		*p*=0.161	*p*<0.001
3 genotype			*p*=0.040
>3 genotype			
Overall model *p*=1.424e-06 & Adjusted R^2 = 0.148			

D	Tank 1	Tank 2	Tank 3
Tank 1		*p*<0.001	*p*=0.982
Tank 2			*p*<0.001
Tank 3			
Overall model *p*= 6.675e-06 Adjusted R^2 = 0.037			

Table 5. 4 Overall survivorship (%) at 12 weeks of genotypes within entity types and across genetic crosses.

	Survivorship 1 genotype entity type (%)	Survivorship 2 genotype entity type (%)	Survivorship 3 genotype entity type (%)	Survivorship >3 genotype entity type (%)	Survivorship total Nº genotypes (%)
AA/AA	13.3	40.5	50.0	47.6	39.6
AM/AM	13.3	12.5	22.2	11.2	14.1
AM/AA	0	36.1	18.0	74.6	50.0

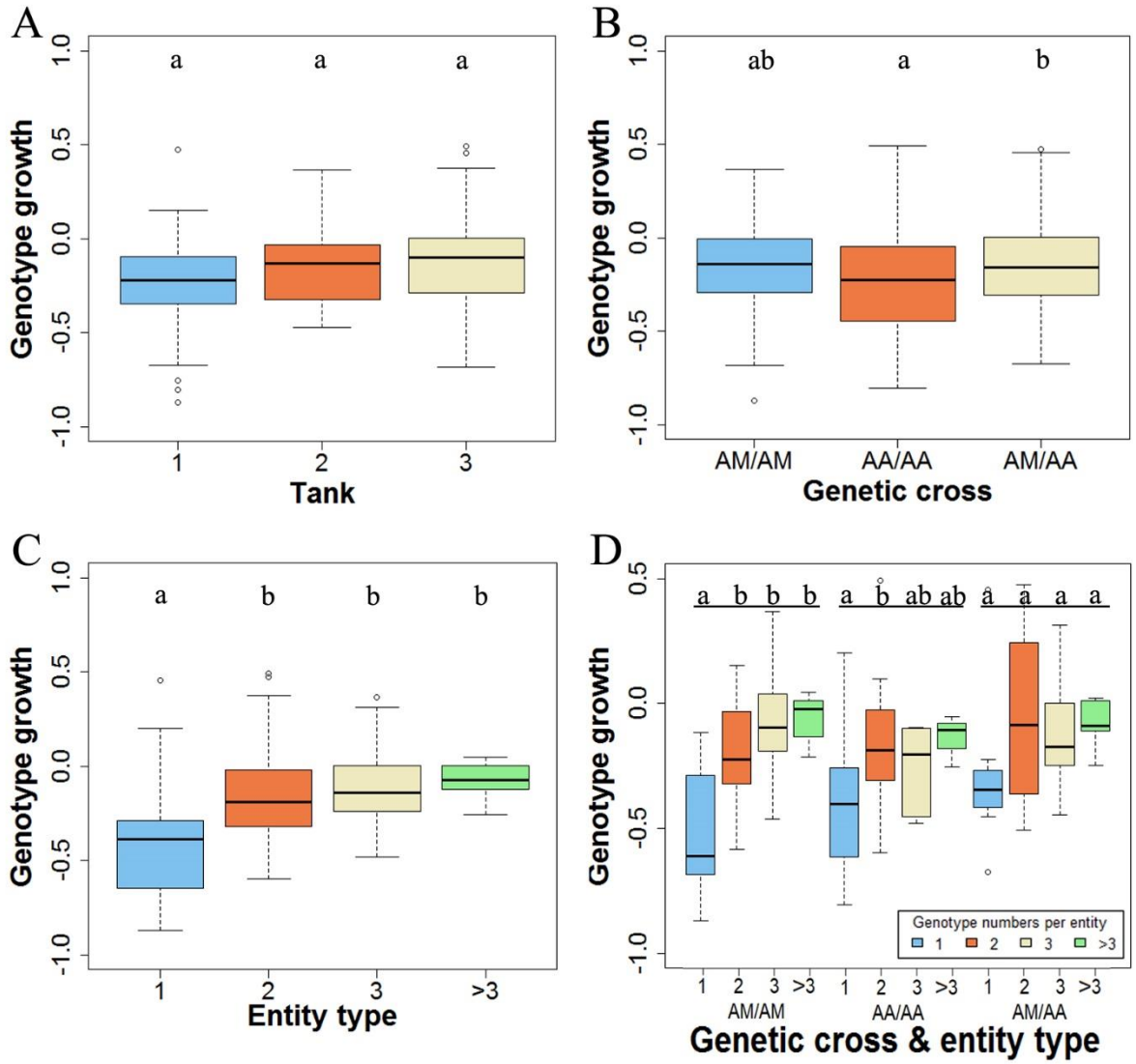


Figure 5. 3 Genotype growth between two to six weeks within three genetic crosses and different entity types.

Pure *Acropora millepora* cross *(*AM/AM), pure *Acropora anthocercis* cross (AA/AA), interspecific hybrid *A millepora/A. anthocercis* (AM/AA), represented as dam / sire. Entity types - 1 genotype, 2 genotypes, 3 genotypes and >3 genotypes. (A) Influence of tank on overall genotype growth with genetic crosses and genotype entities pooled; (B) Genotype growth of three genetic crosses (AM/AM, AA/AA, AM/AA), with entity types pooled; (C) Growth of genotypes within entity types with three genetic crosses pooled; (D) Genotype growth at the entity and genetic cross level. Boxplots show the median, the first and third quartiles, and the lower and upper extremes. Different letters indicate significant differences between growth means,(Linear regression, $p < 0.05$).

Table 5. 5 Results of linear regression of pure genetic crosses of *Acropora millepora* (AM/AM) and *Acropora anthocercis* (AA/AA) and interspecific hybrid *A. millepora*/*A. anthocercis* (AM/AA), represented as dam / sire, on spat growth rates with varying genotype numbers within entities (Entity type - 1 genotype, 2 genotypes, 3 genotypes and >3 genotypes).

(A) Influence on tank effect on growth; (B) Comparison of growth between genetic crosses.; (C) Growth of genotypes within entity types with all genetic crosses pooled; (D) Growth of genotypes within entity types with genetic crosses separated.

A	Tank 1	Tank 2	Tank 3
Tank 1		*p*=0.149	*p*=0.230
Tank 2			*p*=0.792
Tank 3			
Overall model *p*=0.305 & Adjusted R^2 = 0.003			

B	AA/AA	AM/AM	AM/AA
AA/AA		*p=0.131*	*p=0.031*
AM/AM			*p*=0.476
AM/AA			
Overall model *p*=0.091 & Adjusted R^2 = 0.022			

C	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p<0.001*	*p<0.001*	*p<0.001*
2 genotype			*p*= 0.689	*p*=0.375
3 genotype				*p*=0.573
>3 genotype				
Overall model *p*<0.001 & Adjusted R^2 = 0.167				

D – AM/AM	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		*p*=0.001	*p*<0.001	*p*<0.001
2 genotype			*p*=0.078	*p*=0.147
3 genotype				*p*=0.913
>3 genotype				

Overall model $p<0.001$ & Adjusted $R^2 = 0.337$				
AA/AA	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		$p=0.017$	$p=0.258$	$p=0.128$
2 genotype			$p=0.494$	$p=0.942$
3 genotype				$p=0.590$
>3 genotype				
Overall model $p=0.099$ & Adjusted $R^2 = 0.098$				
AM/AA	1 genotype	2 genotype	3 genotype	>3 genotype
1 genotype		$p=0.059$	$p=0.153$	$p=0.096$
2 genotype			$p=0.517$	$p=0.984$
3 genotype				$p=0.599$
>3 genotype				
Overall model $p=0.246$ & Adjusted $R^2 = 0.028$				

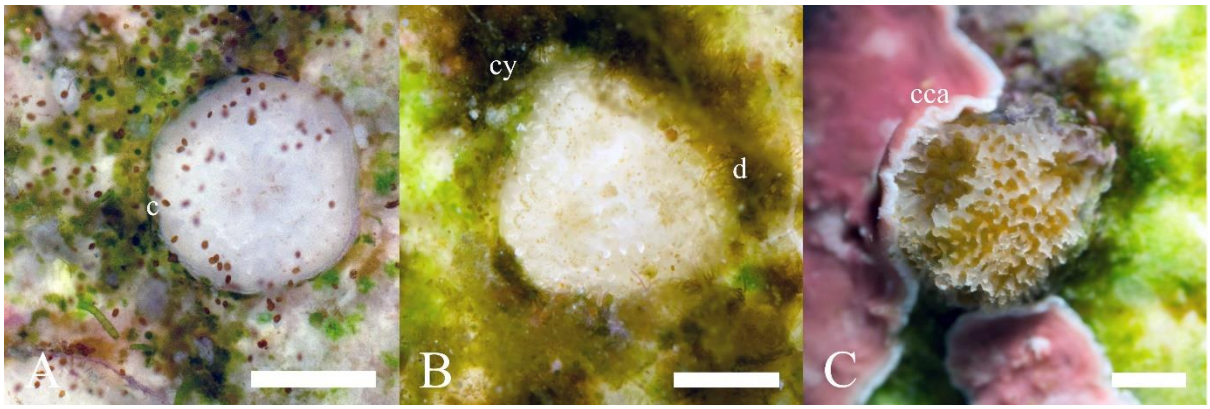


Figure 5. 4 Benthic interactions influencing *Acropora* spat survival.
(A) High numbers of opportunistic ciliates (c) surrounding 2 week post settled *Acropora millepora* spat; (B) Cyanobacteria (cy) and diatom (d) growth around the periphery of 6 week post settled *A. millepora* juvenile; (C) Unknown crustose coralline algae spp overgrowing 12 week post settled *Acropora anthocercis* juvenile. Scale 1mm. *All images taken by J Craggs*

5.6 Discussion

Results indicate that survivorship and growth in post-settlement reef building corals is strongly influenced by the genetic cross and number of genotypes that aggregate within an entity.

The interspecific hybrid of *Acropora millepora* and *Acropora anthocercis* had the highest overall survivorship (50%, Table 5.4) and this was shown to be significantly greater compared to the pure *A. millepora* cross indicating a level of improved survival. While survivorship was also higher in the hybrid compared to the pure *A. anthocercis* cross this was not statistically significant, indicating that hybridisation vigour was not ubiquitous (Chan *et al.*, 2018). As pure crosses of *A. anthocercis* survived better than the pure cross of *A. millepora* the observed increased survivorship in the hybrids may be indicative of inheritance (increase survival) from the *A. anthocercis* paternal route. The second attempt at an interspecific cross with *A. anthocercis* oocytes and *A. millepora* sperm surprisingly failed, which may be indicative of some level of pre-zygotic barriers inhibiting fertilisation success between these species (Wolstenholme, 2004; Wei *et al.*, 2012).

Multi-partner entities, i.e. those with two or more settled spats merged together also exhibited an enhanced survivorship when compared to single entities. More specifically, a peak in optimum survivorship occurred in the pure genetic crosses in 3 genotype entities, although these where not significant compared to 2 genotype entities, but this increased with greater genotype numbers in the interspecific hybrid entities. Such size-mediated survivorship gains are consistent with principles of population biology of colonial organisms (Hughes, 1984; Amar, Chadwick and Rinkevich, 2008), in that the larger the organism at an early stage the greater the probability of survival. A previous study has already highlighted the positive effect that multi genotype aggregations have on survival of reef building corals, i.e. the formation of chimeras during the early post settlement months (Raymundo, 2004). However, such benefits should be limited up to the point of maturation of the allorecognition system, the development of which varies based on the level of sibling relatedness within a settled entity (Puill-Stephan, Willis, *et al.*, 2012). Negative allogenic responses have been shown for non-related *A. millepora* aggregations within three months of settlement, suggesting that this is the time needed for the allorecognition system to mature in *A.millepora*. This might explain (at least in part) some of the observed trends in this study, whereby 'pure crosses' at least in this experiment results in a mix of oocytes and sperm genotypes creating a cohort of full, half and non-related offspring (Puill-Stephan *et al.*, 2012). However, in contrast to the pure crosses, when the interspecific hybrids were produced, the oocytes of a single adult colony of *A. millepora* were crossed with a multi genotype mix of *A. anthocercis* sperm. This was done in order to maximise compatibility and increase the potential for fertilisation success (Edwards *et al.*, 2010). Therefore, offspring of the interspecific hybrid were either fully or half related, and

this may account for the observed increase in survival. As such results should be interpreted within this context of relatedness.

While there were differences in survivorship between the numbers of genotypes growing together and between genetic crosses (pure vs interspecific hybrid), overall survivorship during the 12 week period was low, particularly in the pure *A. millepora* cross (Table 5.4). Survivorship was lower than in other studies in laboratory conditions which reported rates of >80% at 60 days and 67% at 93 days for *A. tenuis* and *A. millepora* respectively (Humanes *et al.*, 2016; Conlan *et al.*, 2017). Such variations highlight the complex interaction that occur on a micro scale that influence survivorship. Indeed adjusted R-squared values within this study indicate that variables outside of the recorded data are strongly influencing overall coral survivorship and growth. Previous research has shown that coral recruits can be affected by predation (Wolf and Nugues, 2013), damage by grazing herbivores (Christiansen *et al.*, 2009; Penin *et al.*, 2011; Trapon *et al.*, 2013) and overgrowth (Birrell, McCook and Willis, 2005; Arnold, Steneck and Mumby, 2010). High densities of ciliates and several species of benthic algae were observed surrounding juvenile coral across all entity types and genetic crosses in this experiment (Fig 5.4). Ciliates have been linked to diseases of many adult corals (Sweet and Séré, 2016) and been shown to predate on recruits in tank settings (Cooper *et al.*, 2007). During the experiment, ciliates and algae were observed to have a negative influence on spat survival, although numbers lost to these two stressors were not recorded throughout. Despite the lack of empirical data in this regard, such competitive benthic interaction, occurring over small spatial scales (mm to centimetres), will undoubtedly play an important role in the success or failure of coral recruitment (Vermeij, 2006). Therefore, the control of these factors via micro-herbivory for example is worth exploring (Villanueva, Baria and Cruz, 2013). Overcoming such bottlenecks in survivorship, or at least increasing the overall survivorship would be instrumental in upscaling reef restoration efforts which are dependent on the grow out of sexually produced coral spat (Guest *et al.*, 2014).

In conclusion, in this Chapter I illustrate that genetic crosses and the number of genotypes within settled entities can influence survivorship over first 12 weeks of life and to a lesser extent growth up to six weeks in post settled broadcast spawning *Acropora* corals. Interspecific hybridisation and size associated with multi-genotype spats can exhibit enhanced survivorship over pure crosses and single genotype settled entities. However when inferring hybridisation

vigour as an explanation for increased survival, further work on understanding the role of relatedness in multi-genotype entities as well as the timing of the initialisation of the allorecognition system in young corals needs to be done in order to give the effects their proper context. Finally, the highly stochastic nature, at a micro scale, of the post-settlement spats' environment requires further research if negative environmental effects are to be controlled during the early stages of coral growth. If possible increased survivorship through such environmental control would underpin upscaling reef restoration practices as well as reducing the costs associated with an individual *ex situ* grown coral.

Chapter 6: *Ex situ* co culturing of the sea urchin, *Mespilia globulus* and the coral *Acropora millepora* enhances early post-settlement survivorship: implications for large scale propagation

This chapter is under review with Scientific Reports as: *Ex situ* co culturing of the sea urchin, *Mespilia globulus* and the coral *Acropora millepora* enhances early post-settlement survivorship: implications for large scale propagation. Craggs, J, Guest, J, Bulling, M, Sweet, M.

6.1 Abstract

Reef restoration efforts that utilise sexual coral propagation need to be up-scaled to have any ecologically meaningful impact. As competitive benthic algae interactions can play a major role in generating post-settlement survival bottlenecks, they need to be addressed, at least as a first step aimed at improving productivity of coral for these initiatives. Sea urchins are keystone grazers in reef ecosystems controlling macroalgal growth. On the other hand, the rasping feeding behaviour of adults can cause substantial physical damage and mortality to developing coral spat. To investigate if microherbivory can be utilised for co-culture I quantitatively assess how varying densities of juvenile sea urchins *Mespilia globulus* (Linnaeus, 1758), reared alongside the coral *Acropora millepora* affected survival and growth of coral recruits. Spawning in both species was induced *ex situ*, ensuring *M. globulus* spawning occurred two months prior to that of *A. millepora.* This effectively ensured an adequate time for larval development, settlement and grow out time. A comparison of *A. millepora* spat reared in three *M. globulus* densities (low 16.67m^{-2}, medium 37.50m^{-2}, high 75.00m^{-2}) and a non-grazed control indicated that coral survival is significantly influenced by grazing activity (p<0.001). Coral survival was highest in the highest density treatment (39.65 ± 10.88%, mean ± sd). Urchin grazing also significantly (p<0.001) influenced coral size (compared to non-grazing control), with colonies in the medium and high-density treatments growing to the largest sizes (21.13± 1.02mm & 20.80± 0.82, mean ± se). Increased urchin density did had a negative influence on urchin growth, a result that is likely to be due to limited food availability.

6.2 Introduction

Anthropogenic driven climate change is causing significant loss of associated biodiversity in coral reef habitats (Richmond, 1993; Pandolfi *et al.*, 2003; Carpenter *et al.*, 2008) resulting in a global decline of these ecosystems (Hughes et al. 2017). This has led some researchers to suggest that human intervention through active restoration will be increasingly important as we move into the third decade of the 21st century (Hoegh-Guldberg *et al.*, 2007; Baums, 2008).

The transplantation of scleractinian corals on damaged reefs has been used widely as a tool for restoration for nearly three decades (Rinkevich, 2005). Transplantation of corals that have been reared from asexually derived fragments is considered a relatively low cost restoration technique and can be implemented with little training (Bowden-Kerby, 2001; Cruz, Villanueva and Baria, 2014). However, this approach has several limitations. For example, the use of asexual fragments results in limited genetic diversity with potentially undesirable consequences such as reduced population resilience when subjected to negative environmental stress (Omori, 2011).

In contrast, production of sexually produced spat circumvents this issue via the genetic recombination and production of new coral genotypes (Baums, 2008; Harrison, 2011). This has led to the development of techniques using sexually reproduced corals for production of transplants, ensuring increased genetic heterogeneity in the transplanted cohort (Omori, 2005; Edwards *et al.*, 2010; Guest *et al.*, 2014). However, this in itself fails to address the larger issues associated with restoration projects which is one of scale. How do we propagate sufficient numbers of corals to counter the loss occurring through anthropogenic degradation? To date *ex situ* sexually reproduced coral spat have only be transplanted onto small areas (i.e. 10s m^2) and the costs of undertaking these approaches remain high. With estimates ranging from US$4.4 to US$60 per coral depending on the method used and the scale of production (Villanueva, Baria and Cruz, 2012; Guest *et al.*, 2014; de la Cruz and Harrison, 2017). There is therefore an urgent need to explore options of up-scaling methods to meet demand. Indeed, new techniques have recently been developed that enable large scale production of sexually produced coral spat for just such scenarios (Pollock *et al.*, 2017). This could then be linked with current, more elaborate attempts around reef restoration including practices such as assisted gene flow (van Oppen *et al.*, 2015, 2017), hybridisation (Chan *et al.*, 2018) and the use of coral probiotics (Peixoto et al. 2017; Sweet et al. 2017). When coupled with the advancements in settlement substrates

(Chamberland *et al.*, 2017), upscaling seems to be within reach technically. However, the cost of such procedures is still argued to be a major hurdle to overcome before reef restoration on a global scale becomes truly feasible. For example, whilst the production of large amounts of planula via in-vitro fertilisation is now possible, upscaling efforts are confounded by the fact that reef building corals undergo a survival bottleneck following fertilisation and embryogenesis, with high mortality rates during the early life history stages, when the newly settled spat are small and vulnerable (Babcock and Mundy, 1996; Wilson and Harrison, 2005; Vermeij and Sandin, 2008; Penin *et al.*, 2010; Guest *et al.*, 2014). Mortality can occur for a broad range of reasons, from chronic stressors such as benthic competition and predation, to more acute impacts associated with bleaching and disease (Richmond and Hunter, 1990). Newly settled coral appear to have a limited ability to deal with competitive benthic interactions (Ritson-Williams *et al.*, 2009). For example, overgrowth by algae (Harriott, 1983; Kuffner *et al.*, 2006) (Fig 6.1 A-C), damage via sedimentation (Birrell, McCook and Willis, 2005) (Fig 6.1 D) and encrusting invertebrates (Harrington, 2004), all regularly negatively impact on survival at this early life stage.

Furthermore, the importance of heterotrophic feeding on spat survivorship has recently been highlighted (especially during early ontogeny), and it has been argued that this aspect needs to be addressed as well if the reduction of mortality rates in these early post settlement stages is going to be possible (Conlan *et al.*, 2017). Therefore, the concept of 'co-culturing' could be utilised to address these issues (Omori, 2005; Omori *et al.*, 2006). Indeed, Villanueva et al. (2013) increased coral spat survivorship when juveniles were co-cultured with the herbivorous gastropod *Trochus niloticus*. Turf algae growth was lowered with increased grazing density and resulted in a 13% increase in spat survivorship when compared to ungrazed surfaces, five weeks post settlement. However, while *T. niloticus* are effective grazers of soft filamentous algae, they are unable to control macroalgae, ruffed algae or crustose coralline algae (CCA) (Lambrinidis, Thinh and Renaud, 1997; Ng *et al.*, 2013). The latter issue could be particularly important when thinking about post settlement survivorship in coral recruits. Whilst CCA and bacteria associated with these algae provide the settlement cue for coral planula larvae to metamorphose from their pelagic to benthic stage (Morse *et al.*, 1996; Heyward, 1999; Negri *et al.*, 2001) many species exhibit varying degrees of anti-settlement strategies such as epithelial shedding, overgrowth (Fig 7.1 E-H), and potential chemical deterrents, all of which have a direct impact of coral post settlement survival (Harrington, 2004).

In the field, coral recruitment clearly increases on reefs where herbivory is high (Edmunds and Carpenter, 2001) and where CCA growth is in check (Bruggemann *et al.*, 1996). However, the size of these herbivores appears to play a key role in coral recruit survivorship (Mumby *et al.*, 2006; Penin *et al.*, 2011; Trapon *et al.*, 2013). Yet there is no experimental work that assesses the influence on growth and survivorship of microherbivory in controlled conditions. Therefore, in this chapter I aimed to firstly assess whether co-culturing the sea urchin *Mespilia globulus* and the hard coral *Acropora millepora* could be achieved, and secondly to test the effect different densities of urchin had on the survivorship of the coral spat.

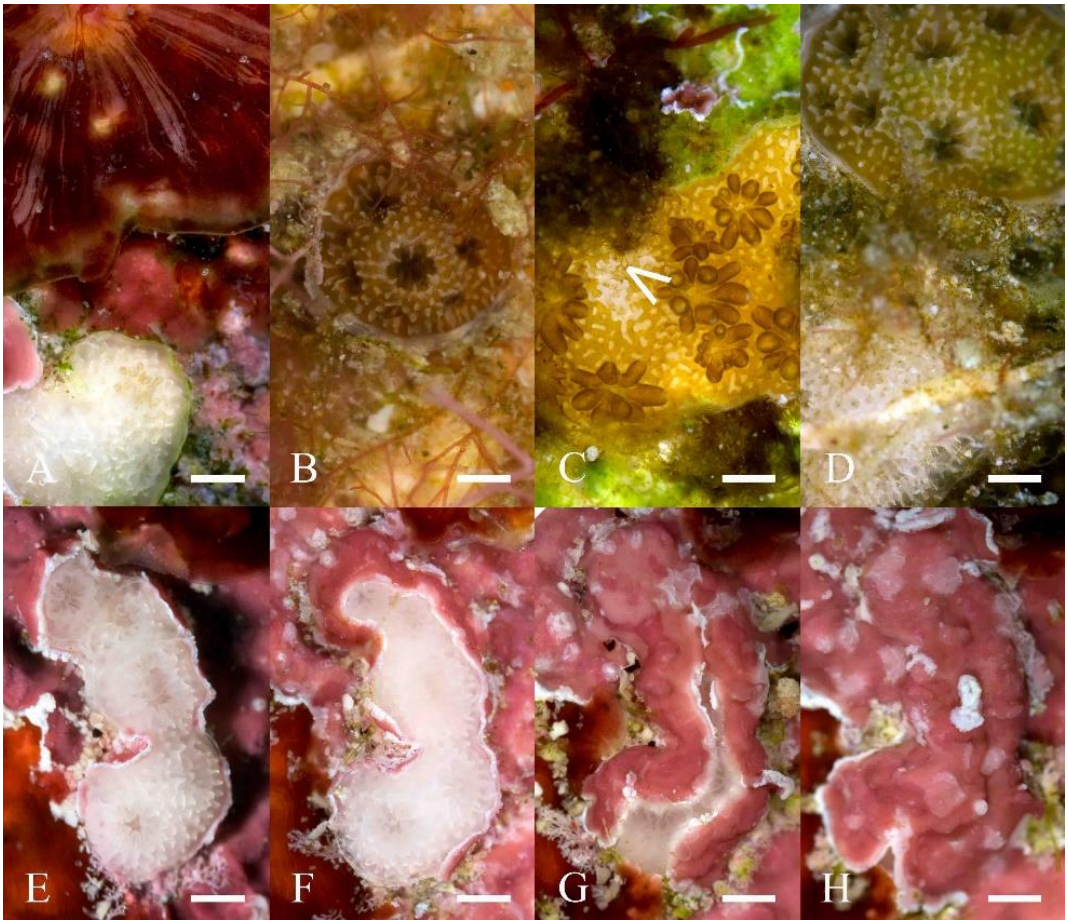


Figure 6. 1 Competitive benthic interactions causing juvenile coral mortality.
(A) *Peyssonnelia squamaria* rapidly over grows juvenile coral; (B) filamentous algae encroaching on *Acropora millepora*; (C) cyanobacteria and diatom growth causing onset of tissue loss (<) in *A. millepora*; (D) sediment accumulation around the peripheral edge of a juvenile *A. millepora*; (E) unidentified crustose coralline algae overgrowing *Acropora hyacthinus* primary polyps on 19/04/16; (F) 25/04/16; (G) 4/05/16; (H) 9/05/16. Scale 1 mm.
All images taken by J Craggs

6.3 Materials and Methods

6.3.1 Co-culturing urchins and corals:

6.3.1.1 Urchin spawning and development.

Ten adult *M. globulus* (19.23 ± 2.03mm, mean diameter ± sd) were housed in aquaria at 27.24 ± 0.83°C (mean ± sd) and salinity of 34.7 ± 1.22ppt (mean ± sd), for three months and fed daily on a diet of dried algae *Porphyra umbilicalis* and *P. yezoensis* (Julian Sprung's SeaVeggies®) and live *Caulerpa prolifera* and *C. brachypus.* Spawning was induced using a rapid temperature change three months prior to the planned *Acropora millepora* spawn (see methods below) ensuring adequate time for urchin development prior to commencing the experiment. All adults were transferred from their holding aquarium to a 20 litre aquarium filled with newly mixed sea water (NMSW). This was prepared with reverse osmosis water mixed to 34.0ppt with solar evaporated sea salt (H2Ocean Pro D&D The Aquarium Solution), and heated to 31.5 ± 0.5 ˚C with a 300 watt aquarium heater (Visitherm). Two male urchins released sperm 8-12 mins after heat treatment commenced and one female released eggs 20 mins later. Gametes were periodically agitated by gently stirring for 45 mins to allow fertilisation to occur (Fig 6.2 A). Ten 1 ml sample counts indicated that a total of 270,000 oocytes were released. Fertilised zygotes were separated into three 16 litre conical culture vessels at an average density of 5.67 larvae ml^{-1}. Developing embryos are negatively buoyant but delicate, and therefore water agitation and aeration was provided with an open-ended airline providing a bubble rate of $5s^{-1}$. This enabled continuous suspension whilst avoiding physical damage. Embryos remained in these cones for the first 72 hrs during which time they received three 50% water exchanges of NMSW by siphoning culture vessel water through a 53 µm mesh ensuring the larvae were left in place.

Embryogenesis was completed three days post fertilisation (Fig 6.2 B-G) and the prismatic larvae (Fig 6.2 H) began to feed on microalgae. To support development of four- to eight-armed echinopluteus (Fig 6.2 I-K) and rudiment development (Fig 6.2 L&M), the algae *Isochrysis* aff. *galbana* Tahitian strain (Haptophyta) was added, as this has been shown to slow larval development time but increase survival rates compared with other algal food sources (Wolcott and Messing, 2005). Larvae were fed at a concentration of 50 cells ml^{-1} at a stocking density of one larva ml^{-1}. *Isochrysis* cultures were grown in three, 5 litre glass demijohns using

Guillard F/2 formulation culture medium (Micro Algae Grow, Florida Aqua Farms). The darkest culture per day was selected for harvesting and algae cell density were determined by ocular microscopy, using a hemacytometer in order to calculate daily larval feed amounts. Following harvesting, cultures were topped up with NMSW at salinity 29-30 ppt and 10 drops per litre of F2 formula were added. Cultures were sieved through a 25 µm mesh (every 3 days) in order to remove particulates, and the demijohns were cleaned with citric acid. Following cleaning they were rinsed with reverse osmosis water and the algae replaced. Aeration was supplied via a 4mm ridged pipe and cultures lit with two 24watt T5 tubes (Plant Pro & Marine white, Arcadia) on a 12:12 light-dark cycle. Larval densities were determined, from 1ml aliquot samples counts, every three to four days in order to monitor population survivorship.

On day 10 (post fertilisation), the larvae were transferred from the conical culture vessel into three, 6 litre kreisel bowls. These bowls had six, 6 cm diameter holes cut into the side (below the water surface), which were covered with a 60 µm filter mesh. The bowls sat in a water bath connected to the same system used for holding the broodstock and an open-ended airline provided gentle water circulation. The meshed holes in the side of the bowls enabled water quality variables (temperature, salinity) inside to match those of the system via diffusion. Once in these bowls the larvae were fed 200 ml of *Isochrysis* twice daily. LED lighting (XHO 50/50, Reef Brite) above the bowls enabled cyanobacteria and diatoms to grow on the internal walls of the bowls, providing food for the juvenile urchins post settlement. Metamorphosis and subsequent settlement occurred from 21 days post fertilisation (Fig 6.2 N-O). Mean diameter of the juvenile urchins (n=186 urchins) was 1.53 mm (SD ± 0.57) 49 days post settlement (Fig 6.2 P) and prior to introduction with settled coral spat.

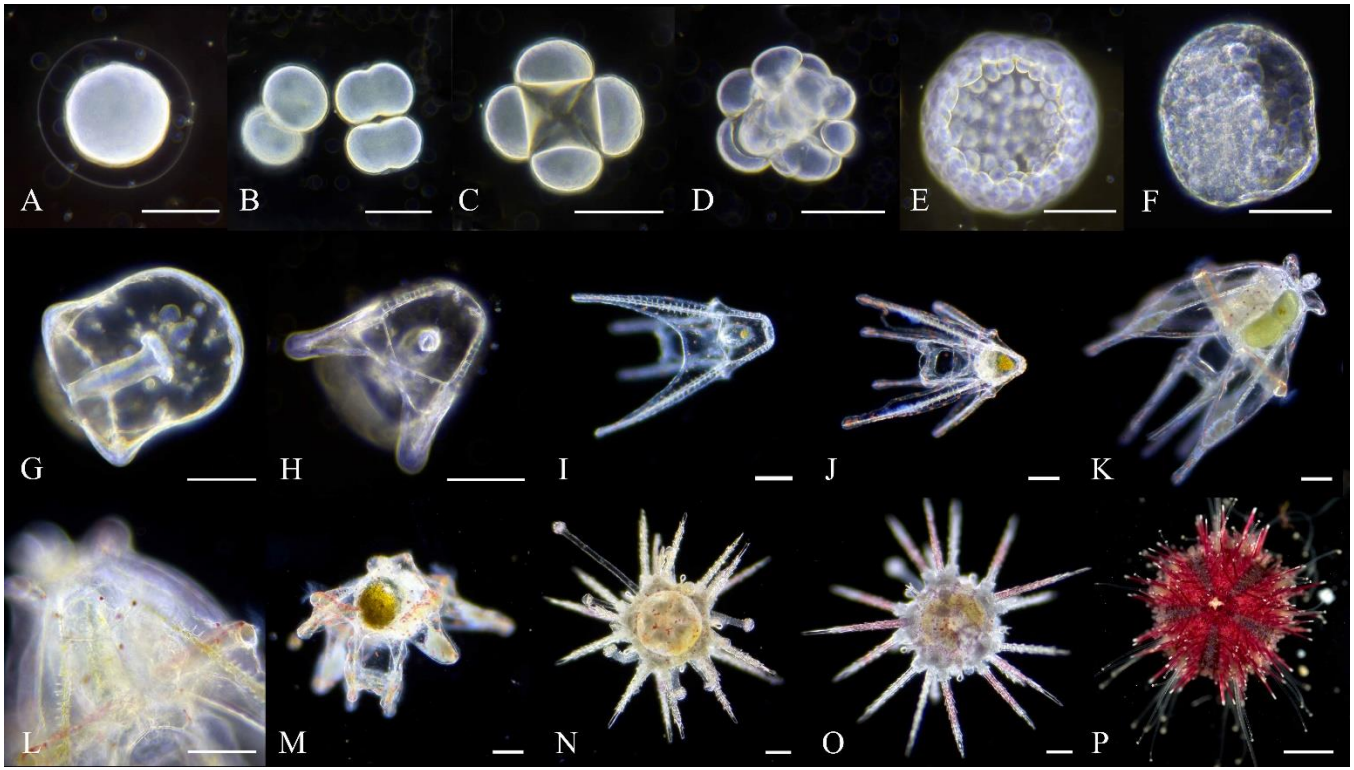


Figure 6. 2 *Mespilia globulus* planktonic development.

(A) Fertilisation membrane surrounding the oocyte 20 mins following oocyte sperm mixing; (B) first cleavage showing two cell blastomere and zygote undergoing early instigation of second cleavage (0.5 hr post fertilisation (hpf); (C) four cell blastomere (1-2hpf); (D) 16 cell blastomere (1-2hpf); (E) blastula (3hpf); (F) cilia have formed and blastula now actively swimming (18 hpf); (G) prismatic stage (20 hpf); (H) two arm echinopluteus stage (25 hpf); (I) four arm echinopluteus stage with ingested *Isochrysis* cells seen in the stomach (3 days post fertilisation (dpf); (J) six arm echinopluteus (11 dpf); (K-L) eight arm echinopluteus 16 & 22 dpf; (M) rudiment formation (22 dpf); (N-P) 1, 2 and 49 days post settlement. Scale A – O = 100µm, P = 1mm. *All images taken by J Craggs*

6.3.1.2 Acropora spawning and development.

A. millepora colonies were conditioned and induced to spawn *ex situ* following the methods described by Craggs et al (2017) and Chapter 3. Full gametogenic cycles were therefore able to be completed and spawning time planned, to within a window of a few days. Artificial lunar cycles were simulated to coincide with natural cycles with full moon occurring on 4th November 2017. Starting from 10th November gamete collection rings were placed directly above eight of the conditioned broodstock colonies and held in place with clips. Thirty minutes prior to the predicted spawning time, the broodstock mesocosm was isolated from the filtration sump below by turning off the main drive pump. In addition, internal water circulation pumps were turned off leaving the water within the mesocosm static. This allowed vertical migration of the buoyant egg sperm bundles (Edwards *et al.*, 2010) and collection within the rings. Five colonies spawned on 14th November 2017 ten NAFM. Following egg sperm bundle release (Fig 6.3 A), gametes from all colonies were mixed, divided into ten 50 ml falcon tubes and spun on a rotator at 10 rpm (Maplelab Scientific, model RM-3). This aided bundle dissociation (Fig 6.3 B). Dissociation was complete when all eggs were separated (46 ± 4 mins ± sd). Following, this the speed on the rotator was reduced to 6 rpm and the tubes left for 45 mins to allow fertilisation to take place. The contents of all tubes were then poured into a 500 ml beaker and the sperm were gravity siphoned leaving the zygotes floating at the surface (Fig 6.3 C). Zygotes were rinsed three times with water from the mesocosm. Following fertilisation embryos were divided into five 6 litre kreisel bowls (described earlier). The bowls sat in a water bath connected to the mesocosm to equilibrate temperature and salinity (27.2 ^{o}C and 34.5 ppt respectively). The water height in the bath allowed the bowls to sit above the water surface, preventing the embryos from being lost, but with the meshed holes below the water surface (Fig 6.4) allowed water exchange via diffusion.

First cellular divisions occurred within two to four hrs (Fig 6.3 D & E). During the first 24 hrs the bowls were left static with no water input (other than the passive diffusion), allowing the embryos to develop past the fragile 'prawn chip' stage (Heyward and Negri, 2012) (Fig. 6.3 H-J). 24 hrs post fertilisation water from the mesocosm system was added to the bowls via a 12 ml silicon hose at a flow rate of 200ml min^{-1}. This allowed water quality to be maintained, but minimised damage over for the remaining time of embryo development (Fig. 6.3 K-N).
The inlet was placed below the water surface and angled to generate a slow circular water movement. If the buoyant developing embryos were dragged down into the water column from the surface the inlet speed was reduced.

Embryogenesis was complete when planula larvae (Fig 6.3 O) were free swimming at 75 hrs post fertilisation and deemed ready to settle. On 17th November 2017 these were harvested from the five kreisel bowls and placed in a bowl filled to exactly six litres with system water from the mesocosm. The water was then randomly stirred with a flat 5 cm × 5 cm piece of UPVC sheet and 10 × 1 ml aliquots were subsequently used to calculate total larval density, which equated to 46,200 larvae. These were introduced to a settlement tank (80 × 40 × 20cm). This tank contained an egg crate sheet housing 494 coral settlement plugs (Ocean Wonders, Ceramic Coral Frag Plugs). The plugs had been preconditioned in the main mesocosm for two months (prior to the experiment commencing) in order to allow a biofilm to grow on the surface. Five open ended air lines provided aeration and water circulation. As discussed above, crustose coralline algae (CCA) aids settlement (Morse *et al.*, 1996). Therefore, a mixture of CCA species (*Hydrolithon* spp, *Sporolithon* sp) were ground in a pestle and mortar with NMSW to make a 50 ml solution. This was then sieved through a 53 µm mesh and added to the settlement tank. In order to maintain suitable water quality during the settlement period the settlement tank received a 50% water change daily by placing a 53 µm plankton collector (Florida Aqua Farms Inc) into the tank and gravity siphoning water from the inside. The tank was topped up back to the same level with water from the main mesocosm. Settlement was completed within seven days of introduction.

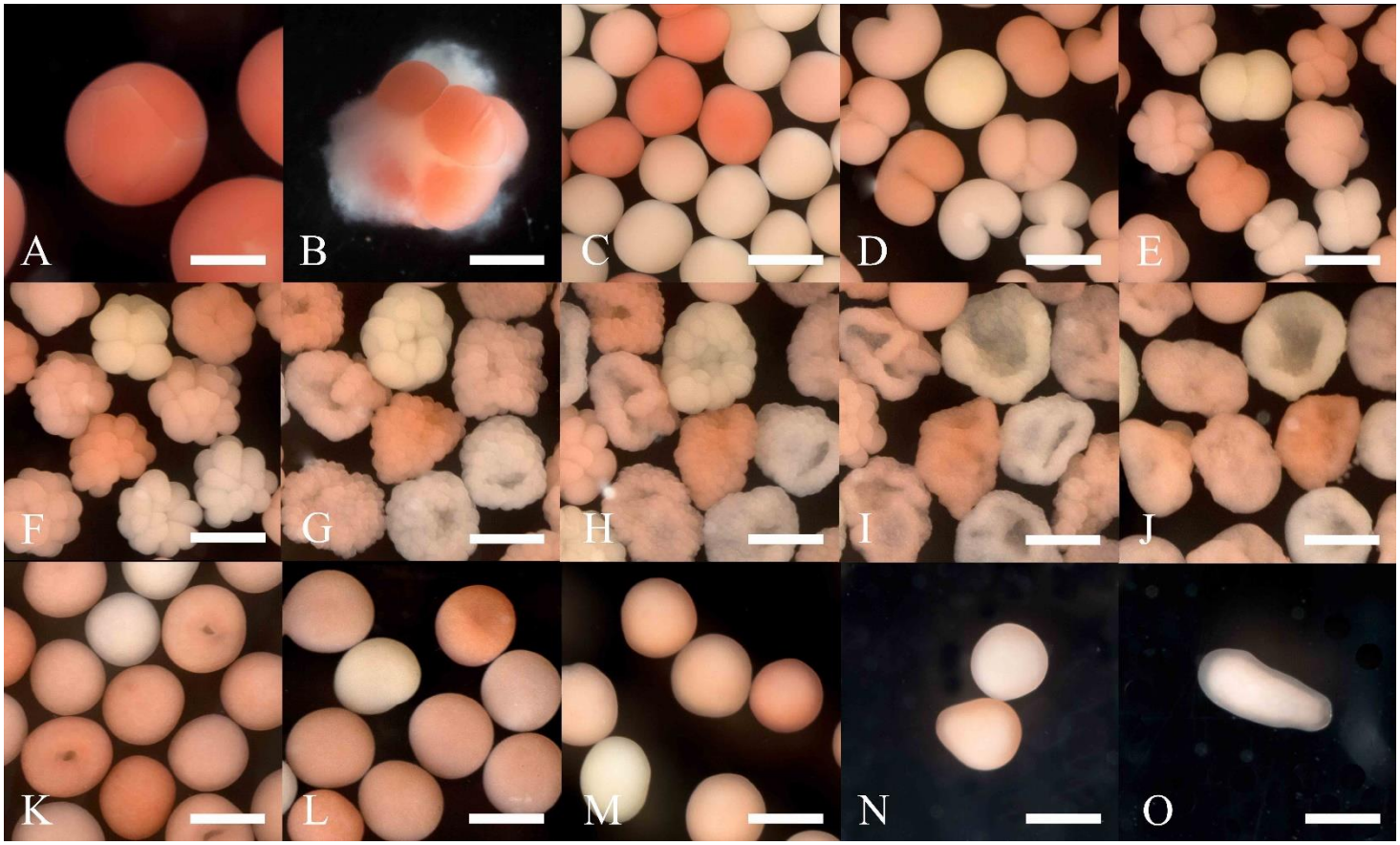


Figure 6. 3 *Acropora millepora* embryogenesis.

(A) Newly released egg sperm bundles; (B) Bundle dissociation occurring, 30-40 mins following release; (C) Zygotes following in-vitro fertilisation; (D) First cell division forming two-blastomere stage, 1-1.5 hr post fertilisation (hpf); (E) Four- and eight-blastomere stage (2-3hpf); (F) Sixteen-blastomere stage (4hpf); (G) Morula stage (5-6hpf); (H-I) 'Prawn chip' stage (6-8hpf); (J) 'Bowl' stage (10hpf); (K-L) 'Round' stage (18-21hpf); (M-N) 'Tear drop' stage (67-70hpf); (O) Planula larval stage (75hpf). Scale = 500µm. *All images taken by J Craggs*

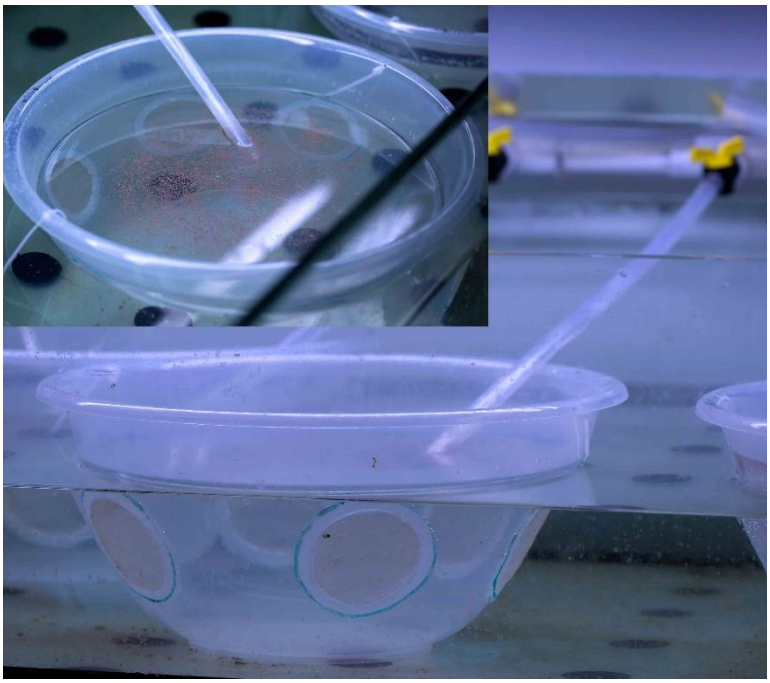


Figure 6. 4 Kreisel bowls used to rear both *Mespilia globulus* larvae and *Acropora millepora* embryos.
The bowls to sit proud of the water surface preventing the loss of larva/embryos. 60 µm meshed holes facilitate maintenance of water quality via diffusion. *All images taken by J Craggs*

6.3.2 Experimental set up:

6.3.2.1 Influence of urchin grazing pressure on coral spat survivorship

Densities of newly settled echinoids of 255 m^{-2} have been recorded on the central Great Barrier Reef (Keesing, Cartwright and Hall, 1993) and peak densities of adults of 73.6 m^{-2} have been recorded in Hawaii (Ogden, Ogden and Abbott, 1989). These were used to gauge the natural grazing pressure coral recruits would experience *in situ*. Four grazing pressure densities were utilised, each with six replicate 10 litre tanks (30 × 20 × 20 cm) (n = 24 in total). Treatments included: non-grazing negative control – consisting of no urchins, low grazing density (4 urchins per tank = 16.67m^{-2}), medium grazing density (9 urchins per tank = 37.50m^{-2}), and high grazing density (18 urchins per tank = 75.00m^{-2}). Following coral planula settlement and initial polyp growth, 18 randomly selected settlement plugs were placed into each of the 24 tanks. Initial numbers of coral primary polyps were recorded, totalling 4826 across all treatments (no

grazing control n = 1225, low grazing n = 1184, medium grazing n = 1220 and high grazing n = 1197). The 24 tanks were housed in three trough style tanks connected to a centralised mesocosm that housed the broodstock coral colonies. Positioning of the replicates of each treatment were randomly generated to ensure a balanced experimental design. Each tank had a banjo style outlet with an 800 µm mesh. Inlet water from the centralised aquarium system was fed at a flow rate of 91.37 ± 6.80 $L.h^{-1}$ into each of the replicate tanks.

Oocytes and larvae of *A. millepora* do not contain symbiotic algae, relying instead on horizontal transmission, and acquiring Symbiodiniaceae from the water column, post settlement (van Oppen *et al.*, 2001; Little, van Oppen and Willis, 2004). To facilitate this, a single two to three cm fragment from the parental colonies was placed into each tank for a period of nine weeks (and then removed). Each tank was cleaned every fortnight to remove algae from the sides and outlet mesh. As scleractinian corals require heterotrophic feeding for optimal survivorship and growth (Petersen, Wietheger and Laterveer, 2008; Conlan *et al.*, 2017) each tank was dosed three times weekly with $0.1ml.L^{-1}$ of amino acid supplement (AcroPower, Two Little Fishes) and live rotifers, *Brachinous plicatilis* (19.15 ±3.13 rotifer / ml), which had been pre enriched with live *Isochrysis* aff. *galbana* Tahitian strain. During feeding, water supply to the treatment tanks was turned off for approximately two hrs. Aeration during this isolation period continued ensuring that rotifers were held in suspension to aid prey capture.

6.3.2.2 Influence of grazing density of coral percentage survivorship

To assess the impact of grazing pressure on spat survivorship, settlement plugs from all replicates were imaged weekly (Canon 5D Mark III with 100mm macro lens) for the 180-day duration of the experiment. Coral polyps for each replicate were counted using ImageJ (Schneider, Rasband and Eliceiri, 2012) and the percentage survivorship for each treatment was calculated based on a comparison with the first week's observation for the corresponding replicate.

6.3.2.3 Influence of grazing density of coral size

Images taken on day 180 for the coral survival percentage counts were also used to measure colony sizes and to assess the influence that urchin density had on coral growth. Coral surface

diameter measurements were taken using ImageJ and the fixed coral settlement plug diameter of 19mm was used as the scale (Fig 6.7).

6.3.2.4 Influence of grazing density on urchin growth

To determine the influence that grazing density had on urchin growth, individuals from each replicate were imaged on day 180 and the body diameter measured using ImageJ. Urchins from each replica were isolated in a 500 ml dish with a 10x10 mm graph paper underneath for scale reference, and imaged three times during the experimental period. The diameter of each urchin (low grazing density – n = 24, medium grazing density – n = 54, high grazing density – n = 108) were determined using the software ImageJ.

6.3.3 Statistical Analysis

Linear regression models (LM) were used to assess coral percentage survival, coral and urchin size, dependent on urchin density (treated as a fixed factor). Residual assumptions homogeneity and normality were assessed using residual diagnostics, and Cook's distances were calculated to identify any overly influential data points following Zuur et al. (2007). Residuals in the model for coral diameter showed heterogeneity of variance and therefore a generalised least square (GLS) extension was applied (Pinheiro & Bates, 2000; West *et al.*, 2007) The most appropriate variance-covariate structure was determined using a combination of AIC scores and plots of fitted values versus residuals for a full model using restricted maximum likelihood (REML). Backwards stepwise selection was applied using maximum likelihood, with the final minimum adequate model being derived using REML.

Kaplan-Meier survival curves were estimated for each treatment. To test for treatment differences between survival curves we conducted pairwise log-rank tests. Finally, differences in proportional risks of mortality between individuals in different treatments were tested using a Cox proportional hazards model.

All analyses were conducted using the statistical programming language R (R Development Core Team, 2015) R version 3.3.0 (2016-05-03). The GLS regression used the nlme package (Pinheiro *et al.*, 2018), and the survival analyses used the survival package (Therneau, 2015).

6.4 Results

6.4.1 Influence of grazing density on coral survivorship

Juvenile urchin grazing had a significant effect on coral survival at day 180 in all grazed treatments (low, medium and high) compared to the non-grazing control at day 180 ($p<0.001$, Fig 6.5, Table 6.1 A). Spat survival was greatest in the highest grazing density (39.65 ± 10.88%, mean ± sd) and lowest in the non-grazed control (5.09 ± 5%) (Fig 6.5 A). Survivorship at high density was significantly greater than low and medium density treatments ($p=0.0099$ and $p=0.0140$ respectively, Table 6.1 A, Fig 6.5 A). No significant difference was observed between corals in the lowest and medium grazing densities ($p=0.877$). The Adjusted R^2 for the model was 0.67.

Pairwise log-rank tests for treatment differences between the Kaplan-Meier coral survival curves (Fig 6.6) showed a similar pattern, with significantly higher survival in all grazed treatments compared to the non-grazed control ($p<0.001$, Table 6.2 A). Coral survival was significantly greater throughout the experiment in the high grazing density compared to both medium and low densities ($p<0.001$ for both high vs low and high vs medium, Table 6.2 A). Again similarly to the linear regression results, no significant difference was found between the Kaplan-Meier coral survival curves between corals in the low and medium densities ($p=0.6$, Table 6.2 A).

Exponent coefficients from the Cox proportional hazards test confirmed that the probability of coral mortality in low, medium and high grazing density treatments were all lower compared to the non-grazed control (0.523, 0.551 & 0.335 respectively, Table 6.2 B). Result indicated that in the highest density treatment, any given coral recruit is approximately a third (0.335) less likely to die than any recruit in the control, non-grazed treatment.

6.4.2 Influence of grazing density of coral size

Residuals for the model of coral diameter showed heterogeneity of variance, and therefore a GLS extension was applied (See Appendix 30 for raw data size frequency distributions). Colony size of corals surviving at 180 days was significantly affected by presence or absence of grazing urchins (Fig 6.5 B, Fig 6.7). Those growing in the presence of urchins all attained a significantly larger size compared to the non-grazed control ($p<0.001$, Table 6.1B). Colony

diameter was also found to be largest in the medium and high grazing treatments and were significantly greater than in the low grazing treatment ($p<0.001$ & $p<0.001$ respectively, Table 6.1 B). Adjusted R-squared were not included for this model as there is no equivalent to R-squared for GLS at present (Table 6.1 B).

6.4.3 Influence of grazing density on urchin growth

Mean body diameter was largest for urchins in the low grazing density treatment (11.01 ± 0.26mm, mean ± se), followed by medium grazing density (7.69 ± 0.17mm). Urchins in the high density treatment grew the least during the 180 day experiment (5.37 ± 0.12 mm) (Fig 6.5 C). Urchin density influenced basal diameter, indicated by significant differences in all pairwise combinations of treatments where urchins were present (low vs medium $p<0.001$, low vs. high $p<0.001$, and medium vs. high $p<0.001$, Fig 6.5 C, Table 6.1 C). The Adjusted R^2 for the model was = 0.70.

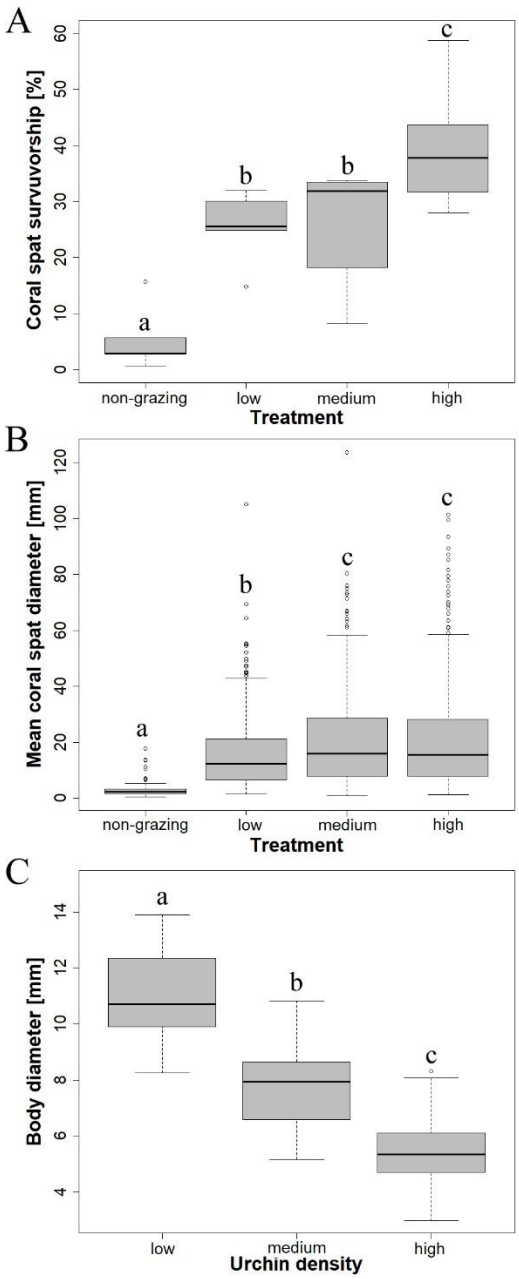


Figure 6. 5 Boxplots of *Acropora millepora* percentage spat survivorship, *A. millepora* spat diameter and *Mespilia globulus* body diameter at 180 days.

(A) *A. millepora* percentage (mean ± se) spat survivorship at 180 days post settlement; (B) *A millepora* spat diameter (mean ± se) at 180 days; (C) *M. globulus* body diameter (mean ± se) at 180 days. (Non-grazing control, low grazing density (four urchins = 16.67 m^{-2}), medium grazing density (nine urchins = 37.50 m^{-2}) and high grazing density (18 urchin = 75 m^{-2}).) The boxplots show the median (black line), the first and third quartiles (grey shaded box), and the

lower and upper extremes, circles represent suspected outlying values. Different letters indicate significant differences between means (Linear regression, $p < 0.05$).

Table 6. 1 Linear regression analysis showing effects of *Mespilia globulus* grazing density on *Acropora millepora* spat survivorship, *A. millepora* colony diameter and *M. globulus* diameter at 180 days

(A) *A. millepora* spat survivorship at 180 days from LM; (B) *A. millepora* colony diameter from linear regression with GLS extension; (C) *M. globulus* basal diameter from LM at 180 days based. (Non-grazing control, low grazing density (four urchins = 16.67 m^{-2}), medium grazing density (nine urchins = 37.50 m^{-2}) and high grazing density (18 urchin = 75.00 m^{-2}).)

A	non-grazing	low	medium	high
non-grazing		*p*<0.001	*p*<0.001	*p*<0.001
low			*p*= 0.8768	*p*=0.0099
medium				*p*=0.0140
high				
Adjusted R^2 = 0.6676				

B	non-grazing	low	medium	high
non-grazing		*p*<0.001	*p*<0.001	*p*<0.001
low			*p*<0.001	*p*<0.001
medium				*p*=0.814
high				

C	low	medium	high
low grazing density		*p*<0.001	*p*<0.001
medium			*p*<0.001
high			
Adjusted R^2 = 0.7009			

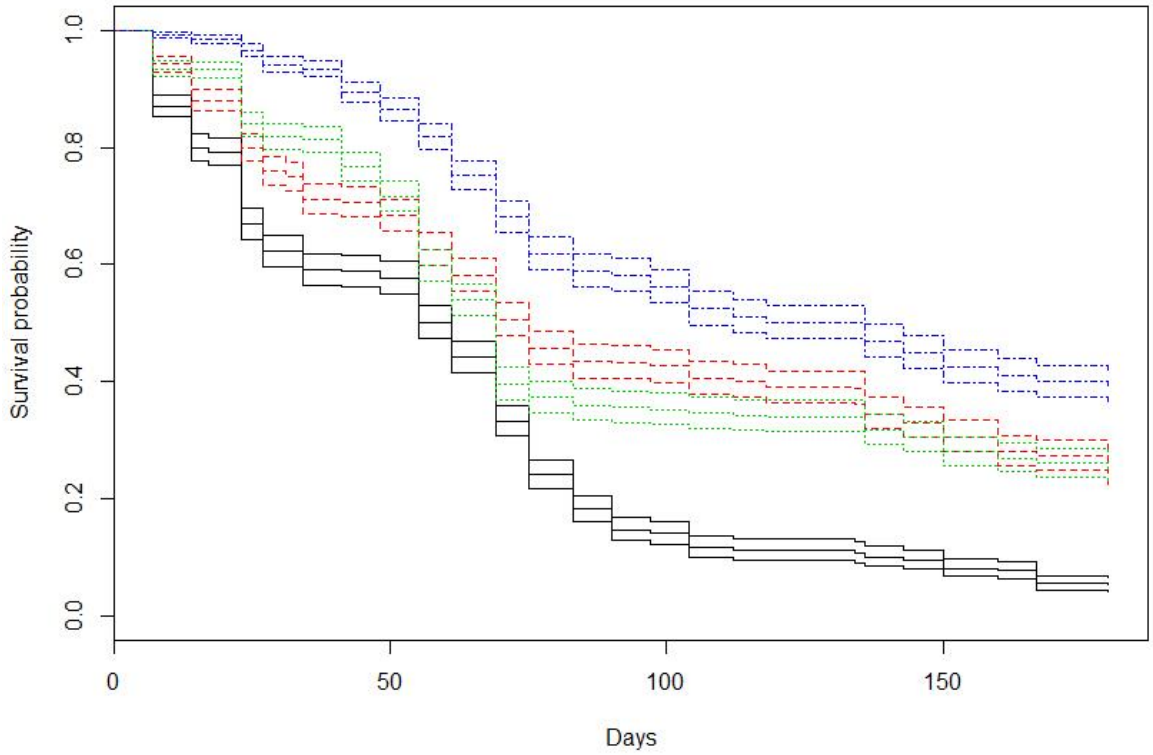


Figure 6. 6 Kaplan-Meier survival curves of newly settled *Acropora millepora* recruits exposed to differing levels of *Mespilia globulus* grazing pressures.
(Non-grazing control (black solid line), low grazing density four juvenile *M. globulus* (16.67 m^{-2}) (green dotted line), medium grazing density nine juvenile *M. globulus* (37.50 m^{-2}) (red dashed line), high grazing density 18 juvenile *M. globulus* (75.00 m^{-2}) (blue dash dot line)) and grown over 180 days (mean ± se).

Table 6. 2 Kaplan-Meier survival showing effects of *Mespilia globulus* grazing density on *Acropora millepora* spat survivorship over 180 days.
(A) Pairwise log-rank tests differences between treatment survival curves; (B) Cox proportional hazard models exponent coefficient and (significance) of differences in proportional risks of mortality between individuals in different treatments. (Non-grazing control, low grazing density (four urchins = 16.67 m^{-2}), medium grazing density (nine urchins = 37.50 m^{-2}) and high grazing density (18 urchin = 75.00 m^{-2}).)

A	non-grazing	low grazing density	medium grazing density	high grazing density
non-grazing		$p<0.001$	$p<0.001$	$p<0.001$
low grazing density			$p=0.6$	$p<0.001$
medium grazing density				$p<0.001$
high grazing density				

B	low grazing density	medium grazing density	high grazing density
non-grazing	0.5232 ($p<0.001$)	0.5512 ($p<0.001$)	0.3351 ($p<0.001$)

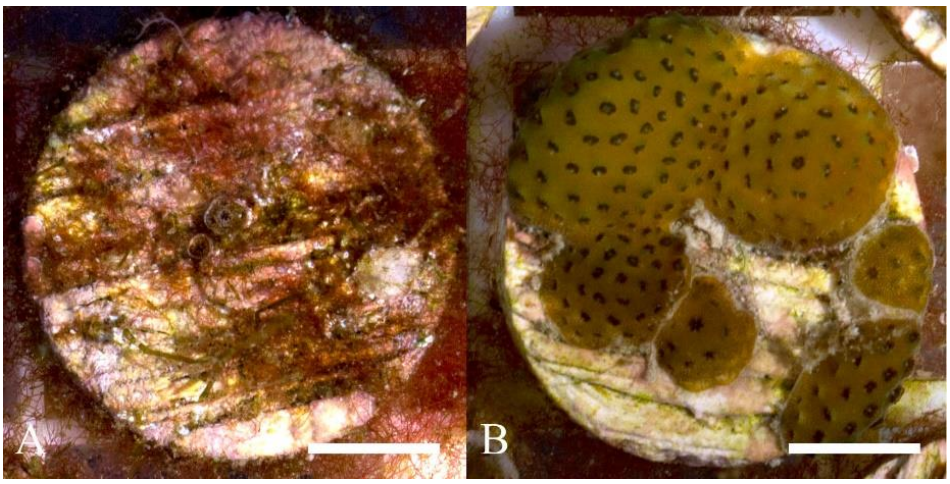


Figure 6. 7 Comparison of *Acropora millepora* colonies size at 180 days between treatments. (A) non-grazing control and (B) high urchin density (75 urchins m^{-2}). Scale = 5mm. *All images taken by J Craggs*

6.5 Discussion

Upscaling restoration efforts must focus on improved productivity, improved coral resilience and increased yield (van Oppen *et al.*, 2017). Increasing coral survivorship during early propagation is a critical first step as this should dramatically reduce the cost associated with any given coral transplanted back onto the reef. Here the sea urchin *Mespilia globulus* can be induced to spawn on demand using rapid temperature shock and, with appropriate planning,

reared and grown (*ex situ*) for utilisation as an effective grazing species in co-culture with coral spat. Furthermore, during co-culturing urchin density was shown to play an important role in modulating coral survivorship and growth rates. Both species can be reared using low 'tech' transferable methodology with a high level of success, the highest survival rate being 39.65% or 475 *Acropora millepora* colonies and more than 200 juvenile *M. globulus* from a single spawning event.

Increased urchin density resulted in significantly higher coral survivorship over the 180 day period, probably due to the reduced benthic competition from algal species with results being comparable to similar field based studies with the gastropod mollusc *Trochus niloticus* (Villanueva, Baria and Cruz, 2013). Although Villanueva et al. (2013) also highlighted improved coral survivorship at higher grazing densities (eight *T. niloticus* m^{-2}), their study only spanned five weeks (35 days). Furthermore, survivorship was higher in the current study utilising *M. globulus* (39.65 ± 10.88%, mean ± sd) compared to that shown when using *T. niloticus* as the grazer (18.3% ± 6.7%, mean ± sd), suggesting urchins are possibly a more effective grazing invertebrate when the goal is increasing coral survivorship.

Furthermore, increasing urchin density not only enhanced coral survivorship but also resulted in increased coral growth rates. This was likely to be a result of microherbivory reducing competition with benthic algae and allowing unimpeded coral growth, a result previously unreported. As larger corals have a greater survivorship, once out planted on the reef (Guest *et al.*, 2014), increasing growth rate allows for out planting sooner, reducing restoration costs in this area.

Unsurprisingly, whilst coral survival and size were positively affected by increasing urchin density, these benefits appeared to come to the detriment of the urchins. As density increased, urchin growth rate was reduced, probably as a result of limited food availability (Ebert, 1968). What was surprising is that, despite a detailed assessment of the health of the recruits, there was no evidence of physical abrasive damage to the corals, which was expected due to the rasping actions of urchin grazing. This suggests that survival was enhanced due to the smaller sizes of the urchins i.e. the co-culturing methodology was effective for the corals as the urchins were too small to significantly damage the corals whilst grazing on the algae.

This study illustrates that a more holistic approach of multi-taxa co-culturing can increase the production of sexually diverse coral spat and that if applied to restoration practises, could

facilitate up-scaling efforts. The period over which a coral remains in a nursey (prior to transplantation) influences growth, survival and ultimately cost per unit of the transplant, with longer nursery rearing periods leading to lower overall costs per coral (Guest *et al.*, 2014). Time invested in nursery care therefore plays an important role in the cost per unit effort and methods to increase production must be an important focus in reducing overall costs of restoration efforts.

The practice of co-culturing organisms (spanning different trophic levels) has been undertaken before, in bioremediation of coastal aquaculture initiatives (Pierri, Fanelli and Giangrande, 2006) for example. Lower trophic organisms such as the red alga *Gracilariopsis lemaneiformis* utilise the waste products, inorganic nitrogen and phosphorus, from higher trophic target species (such as the food fish *Sebastodes fuscescens*) (Zhou *et al.*, 2006). This is in order to reduce negative environmental impact associated with eutrophication (Zhou *et al.*, 2006). Reid et al. (2011) highlighted that economic value increased when an integrated multi-trophic aquaculture approach was utilised. Such multi-trophic approaches are now clearly achievable with regard to coral reef restoration practices. For example, an early study by Pomeroy et al. (2006) investigated the financial and social feasibility of aquaculture of a variety of reef organisms, as an alternative to wild collection. Through analysis of socioeconomic dimensions they concluded that, under certain conditions, such an approach could provide alternative livelihoods to local people and prove useful in reducing fishing pressure on the reefs. The methods illustrated in this study should open up an avenue for self-sustained funding if the co-cultured organisms (urchins in this instance), also have an economic value themselves (Rhyne *et al.*, 2012). The coral *A. millepora* and the sea urchin *M. globulus*, utilised in this study, are regularly imported across the marine ornamental industry. Therefore, increasing *ex situ* culturing of these organisms should reduce demand on wild stocks, with the added benefit of supplementing reef restoration practices as well.

Targeting more economically important species in any such co-culturing venture would further support cost reduction. For example, the collector sea urchin *Tripneustes gratilla* (Linnaeus, 1758) is an important food item in many countries across the world such as the Philippines and South Korea (Toha *et al.*, 2017). Whilst not the main focus of this chapter *T. gratilla* has now been successfully cultured and the embryo and larval developmental stages documented (Fig 6.8), which illustrates the suitability of this species as an alternative grazer to *M. globulus*. Once juveniles have surpassed their 'useful size' for improved spat survivorship they could be grown on for the secondary process of roe production (Mos *et al.*, 2011).

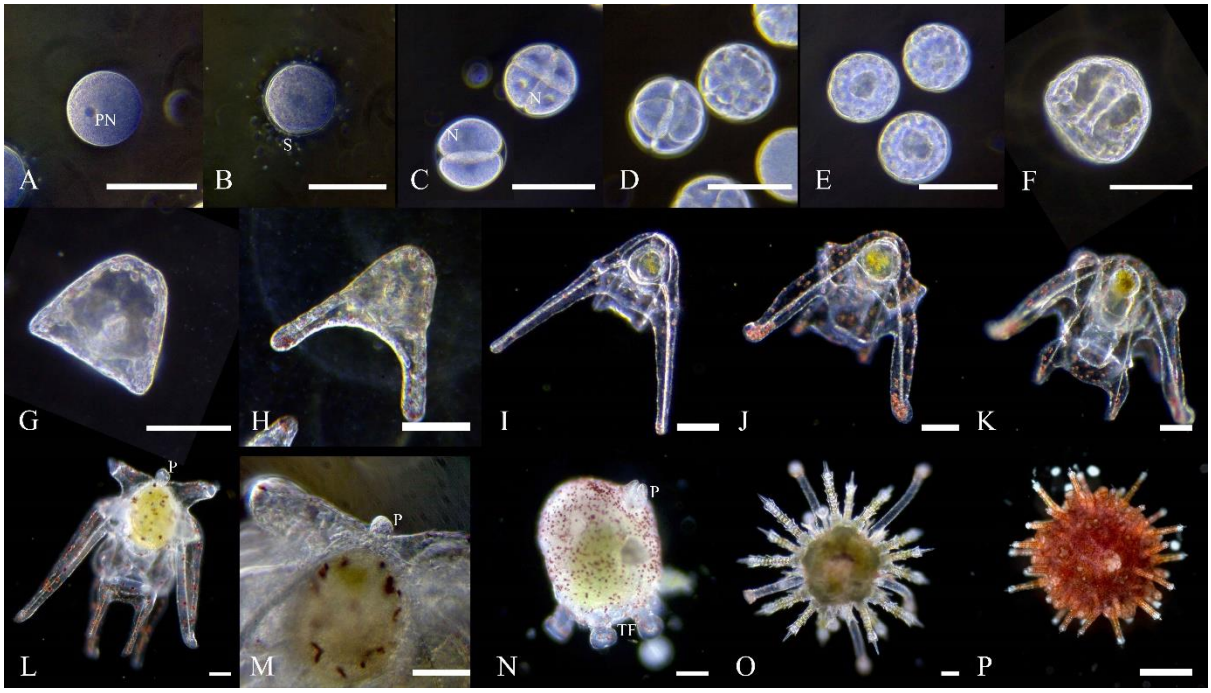


Figure 6. 8 *Tripneustes gratilla* planktonic development.

(A) Newly released oocyte showing pronucleus (PN); (B) Spermatozoa surrounding the oocyte 35 mins following oocyte sperm mixing; (C) First and second cleavage, two- and four-cell blastomere with nucleic visible (N) (0.5 – 1.5 hr post fertilisation (hpf); (D) Eight- and 16-cell blastomeres (2 hpf); (E) Blastula (4.5 hpf); (F) Cilia have formed, blastula now actively swimming (18 hpf); (G) Prismatic stage (20 hpf); (H) Two arm echinopluteus stage (2 days post fertilisation, dpf); (I) Four arm echinopluteus stage. Ingested *Isochrysis galbana* cells can be seen in the stomach (5 days post fertilisation (dpf); (J) Initialisation of six arm echinopluteus stage (11 dpf); (K) Six arm echinopluteus (12 dpf); (L) Eight arm echinopluteus, with emergence of the first pedicellaria (p) (21 dpf); (M) Enlargement of Figure 7.8 L showing first pedicellaria (p) (21 dpf); (N) Newly settled 'echinoporculus' stage, an intermediate stage between the larvae and juvenile. 1 day post settlement (dps); (O) metamorphosed juvenile (7 dps); (P) Juvenile (40 dps). Scale A – O = 100µm, P = 1mm. *All images taken by J Craggs*

Another possibility is for endangered coral and/or urchin species to be utilised in the co-culturing. In the Caribbean for example, two coral species *Acropora palmata* and *Acropora cervicornis*, both listed as Critically Endangered by the IUCN Red List of Threatened Species, along with the key stone herbivore species *Diadema antillarum* (Philippi, 1845), suffered massive declines in populations during the early 1980s (Lessios *et al.*, 1984; Porter and Meier, 1992; Gardner *et al.*, 2003). These three species are therefore prime candidates for co-culturing efforts. To date, only limited success has been had in restoration efforts for these species but *ex situ* rearing has been shown to be possible for both the corals and the urchins separately (Idrisi, Capo and Serafy, 2003; Quinn and Kojis, 2006; Vaughan, 2010; Young, Schopmeyer and Lirman, 2012; et al, 2015), highlighting the promise of future work.

6.6 Conclusion

In conclusion, in this Chapter I show that microherbivory can play an important role in increasing coral survival and growth during early ontogeny. The co-culturing methodology used offers significant potential for future coral conservation efforts as it combines enhancing survivorship of coral for transplantation with opportunities to develop sustainable alternative livelihoods and/or support the conservation of threatened urchin species. Future work is now needed to build towards using these methods at larger scales in order to make significant contributions to coral conservation.

Chapter 7: Three years to complete the life cycle of *Acropora millepora* in a closed mesocosm

This chapter is under review with Marine Biodiversity Oceanarium; **Craggs J**, Guest JR, Davis M, Sweet M. Three years to complete the life cycle of *Acropora millepora* in a closed mesocosm.

The ability to close life cycles in order to produce multiple filial generations (F1 & F2), from known parental crosses, under controlled conditions, provides opportunities to study heritability of phenotypic traits (van Oppen *et al.*, 2015). Tropical corals are keystone habitat forming species and their reproductive biology has been a topic of intense research for more than three decades (Baird, Guest and Willis, 2009). Reports detail corals being reared from eggs to spawning adults *in situ* (e.g. Baria et al. 2012), but to date none have completed the life cycle and produced an F2 generation of a broadcasting coral in fully closed mesocosms. Recent advances in *ex situ* mesocosm design (Craggs et al. 2017, Chapter 3) allow gametogenic cycles of broadcast spawning corals to be completed and here I report the first successful production of an F2 generation in *A. millepora*. Spawning of broodstock colonies (P), originally collected from the Great Barrier Reef, Australia occurred at the Horniman Museum and Gardens, London on 7th December 2015 (Fig 7.1 A). Following *in vitro* fertilisation, six surviving colonies (Fig 7.1 B, juvenile at 7 months old) were grown-out for three years. In 2018, two of these were sexually mature, containing pigmented oocytes (Fig 7.1 C, oo). Onset of sexual maturity is believed to be controlled by polyp age and colony size, with three-year-old sexually propagated colonies of *A. millepora* spawning at greater than12.3 cm in diameter (Baria *et al.*, 2012). In this study colonies, also three years old at spawning, had diameters of 9.9 and 11.6 cm, whilst the four non-gravid colonies had diameters between 5.7 and 9.4 cm. The spawning of F1 corals synchronised with wild colonies on the GBR (based on spawning observations at the Australian Institute of Marine Science, National Sea Simulator as the proxy for wild), (21.00-21.15 on 27th and 28th November 2018) (Fig 7.1 D). Gametes were collected and cross fertilisation performed to produce the F2 generation. Mean fertilisation rates (89.74 ± 0.35% SD) (Fig 7.1 E) and (95.64 ± 0.97% SD) (Fig 7.1 F), were comparable to those previously recorded from wild crosses (Humphrey *et al.*, 2008).

Such *ex situ* spawning offers substantial cost cutting advantages as well as reducing reliance on *in situ* spawning and collection of broodstock from dwindling wild populations. This

breakthrough will thus support the development of the field of coral biology and further research into *ex situ* mesocosm designs and husbandry approaches can build on this foundation.

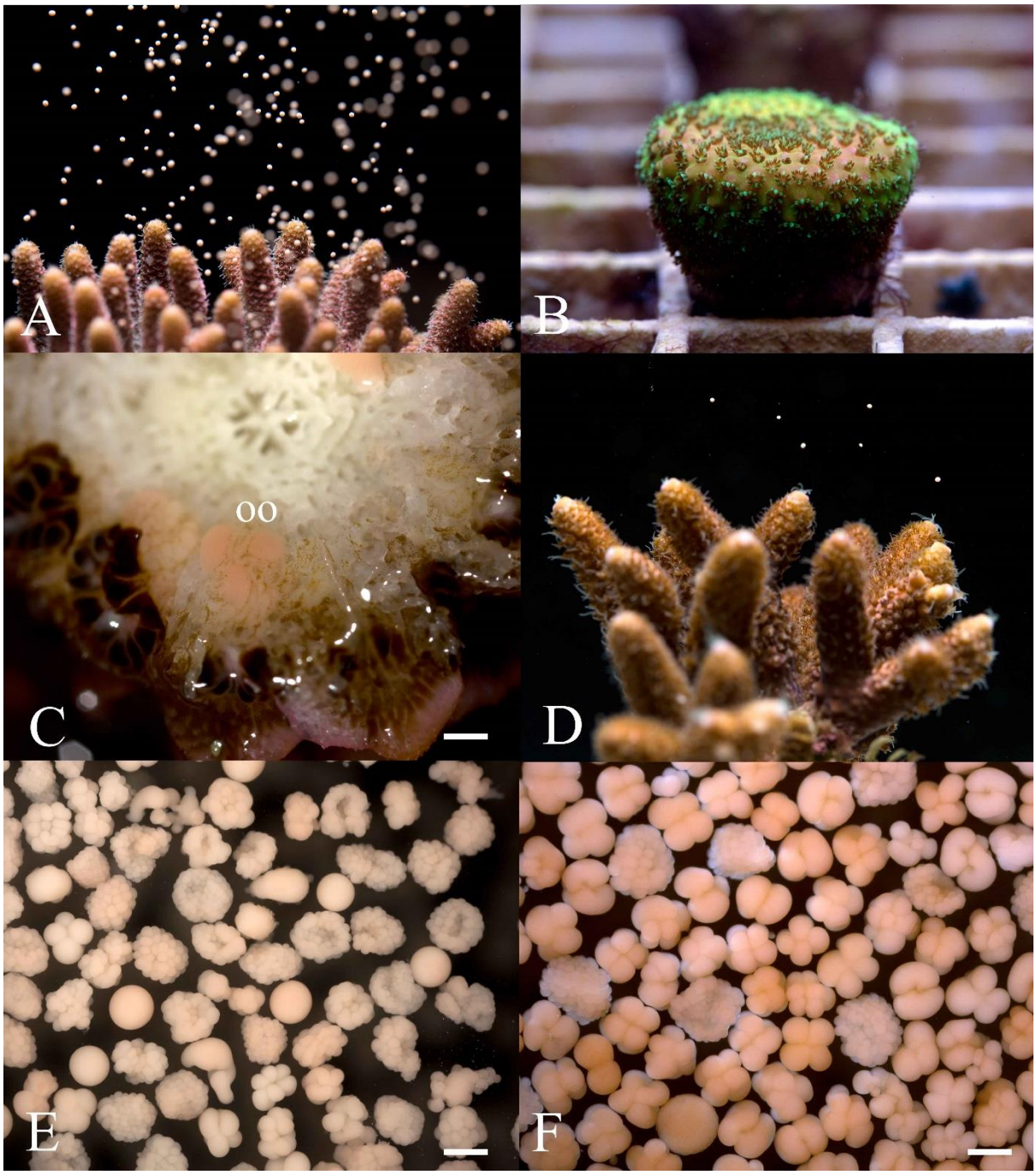


Figure 7. 1 Closing the life cycle of *Acropora millepora ex situ*.
(A) Broodstock colony spawning *ex situ*; (B) Seven-month-old F1 colony; (C) Three-year-old F1 colony with pigmented oocytes (oo); (D) Three-year-old F1 colony spawning *ex situ*; (E & F) F2 embryos from F1 pair wise crosses. Scale = 1mm. *All images taken by J Craggs*

Chapter 8: General discussion of thesis findings and future directions

8.1 Introduction

The purpose of this thesis was to develop novel approaches to induce broadcast spawning in various scleractinian coral species, to support reproduction in a planned and predictable manner within *ex situ* environments. Following the developmental phase, gametes produced were then used to investigate methods of increasing post settlement survivorship. The broader aim was to facilitate *ex situ* spawning supporting reef restoration practises through land based coral nurseries, and to therefore provide a new option for the coral reef restoration 'tool box' for the future.

8.1.1 Background to work associated with the thesis

Work focusing on sexual reproduction of broadcast spawning corals commenced at the Horniman Museum and Gardens in 2012 with the initial goal of stimulating broadcast spawning in *Acropora* species in a planned and predictable way in aquarium environments. This initial work focused on five parameters, which at the time, I believed would be important to achieve this goal; the size of a colony, amount of heterotrophic feeding, seasonal temperature change, photoperiod and the lunar cycle.

Initially two species were used; *Acropora valida* (Dana, 1846) and *Acropora prostrata* (Dana, 1846). At first, I worked under the assumption that colony size is an important precursor for reproduction. *A. valida* had been reported to spawn at a mean diameter of 50 cm (Nosratpour, 2008) and *A. prostrata* had been observed spawning *in situ* at approximately 40 cm diameter (Pers Obs Jamie Craggs). As such, I developed broodstock colonies by 'combining' multiple single genotype fragments (Fig 8.1 A) of each species into a single large piece. This was undertaken by placing the single fragments onto one piece of liverock using a two-part epoxy putty (Milliput Standard yellow/grey) (Fig 8.1 B). Using the previously mentioned diameters as a guide (Fig 8.1 C), fragments were glued with the view that small pieces could grow, and via isogenic fusion (Fig 8.1 D&E), create colonies that were of a reproductive size more rapidly than waiting for single small fragments to grow (Fig 8.1 F).

A modular research system that had previously been used for coral disease experimentation was used to complete initial trials (Fig 8.2 A). This system was adapted using simple building materials to construct a black out system to eliminate external light pollution and facilitate full control of the light/dark environment *ex situ* (Fig 8.2 B). Attached to this adapted research system was a microprocessor (GHL, Proflix), into which seasonal temperature, photoperiod and lunar cycle data, sourced from Bequ Fiji were programmed.

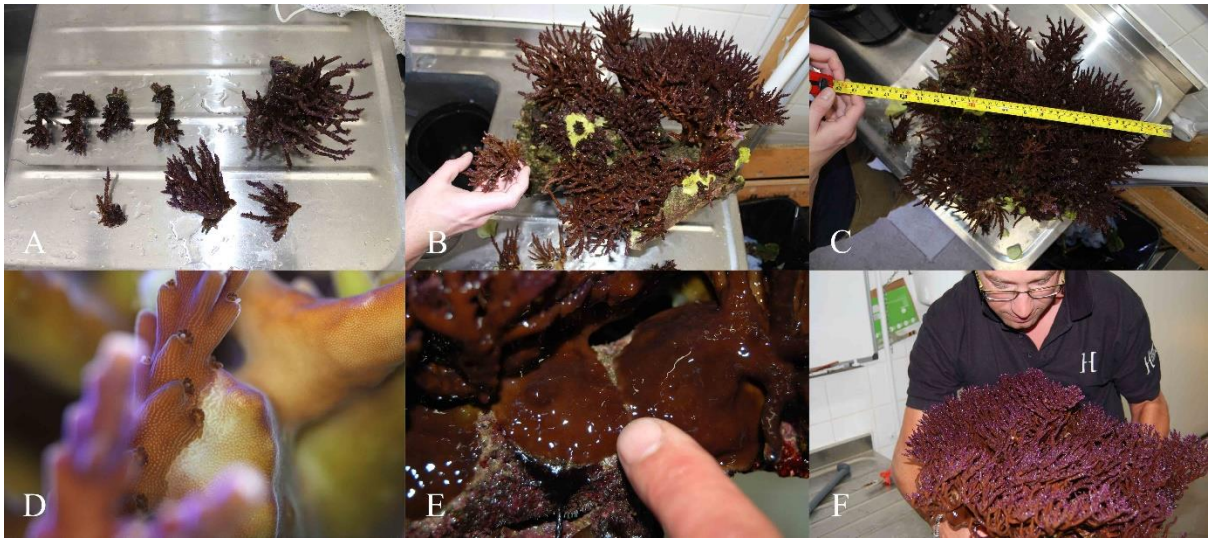


Figure 8. 1 Construction of *Acropora valida* broodstock colony for *ex situ* broadcast spawning research.
(A) Multiple fragments of the same genotype are collected from public display tanks at the Horniman Museum and Gardens; (B) Fragments being attached with a two-part epoxy to a single large piece of liverock; (C) Constructed colony measures 50 cm in diameter; (D & E) Via isogenic fusion fragments grow into each other; (E) The completed broodstock colony eight months after it was constructed.

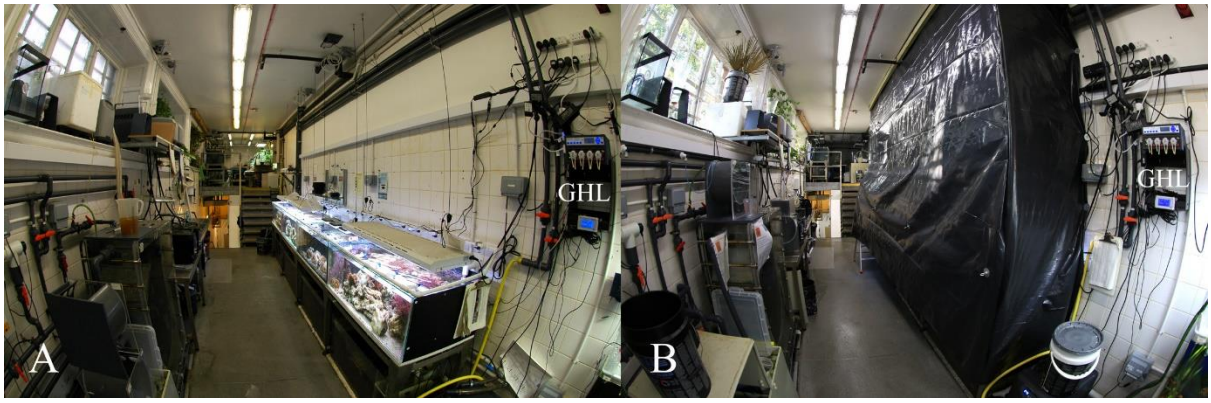


Figure 8. 2 Initial coral research system at Horniman Museum and Gardens modified to support broadcast spawning research.
(A) Four tank modular coral research system, with GHL microprocessor (GHL) attached, behind the scenes of the aquarium; (B) Using cheap building materials black out system enables the light dark environment to be fully controlled *ex situ* and eliminates light pollution.

8.1.2 First broadcast spawning

The formation of the two colonies and adaptation of the research system was completed in December 2012 and by August 2013 both colonies had developed oocytes, with the *A.valida* one month ahead of *A. prostrata*. *A. valida* spawned on 6th September 2013, 16 NAFM and the following month *A. prostrata* spawned on 6th October 2013, 17 NAFM.

This initial experiment highlighted the potential of *ex situ* broadcast spawning and was well regarded within the aquarium community. However, the work was more of a trial rather than a robust scientific approach. Therefore, this thesis aimed to take what was fundamentally a husbandry-based project and make it scientifically relevant, which in turn might allow *ex situ* spawning to be developed as a new tool for applied coral research and reef restoration. In addition this thesis aimed to develop approaches to increase post settlement survival and provide a broader understanding of pressures that drive high mortality in early ontogeny.

Below, I focus on each chapter in turn, highlighting successes, new questions that have arisen during the course of the three years, and identify potential methodological improvements.

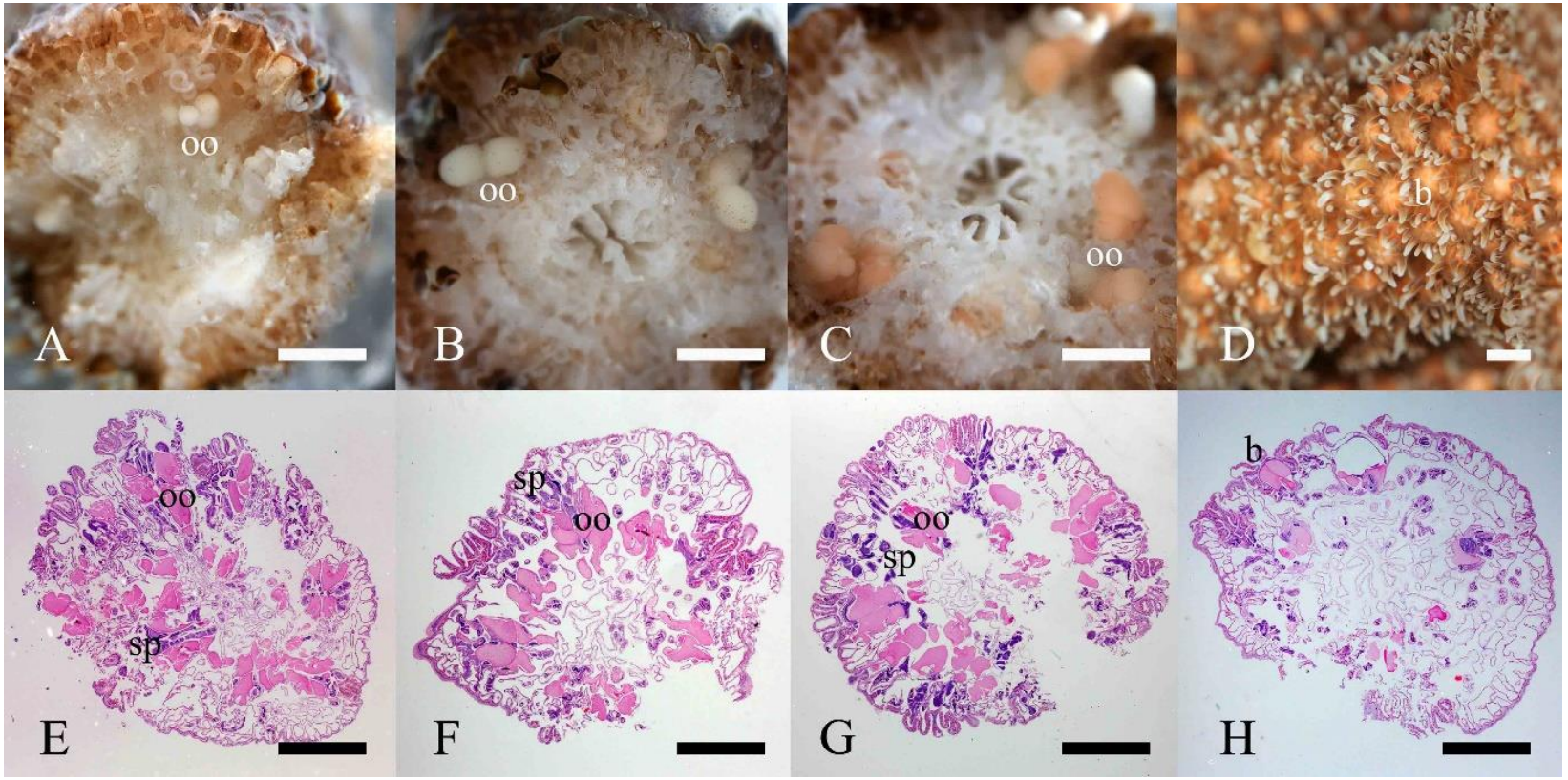


Figure 8. 3 *Ex situ* gamete development in *Acropora prostrata*.

(A – C) Fragment cross section showing oocytes development; (E-H) Haemotoxylin and Eosin stained histological sections; (A&E) Early stage of oocyte and sperm development, 13th August 2013; (B&F) Large white mid stage oocytes, 25th August 2013; (C&G) Pigmented oocytes, 16th September 2013; (D&H) Colony setting prior to *ex situ* gamete release, 6th October 2013 Oocytes (oo), Sperm (sp), egg sperm bundle (b). Scale = 1 mm. *All images taken by J Craggs*

8.2 Chapter 2: Maintaining natural spawning timing in *Acropora* corals following long distance inter-continental transportation

Few studies have documented the transportation of live scleractinian coral (Petersen *et al.*, 2004; Delbeek, 2008) and to date none have focused on the feasibility of shipping gravid colonies. The aim of this chapter was to describe a method to transport large gravid *Acropora hyacinthus* colony fragments from Singapore to London in order to establish broodstock colonies, known to be of a sexually mature age. In addition, the study tested whether, following long distance intercontinental travel, colonies would release their gametes in synchrony with wild counterparts on their natal reefs in Singapore. At the time of collection, 12 out of 14 colonies contained large oocytes and these spawned within the same lunar month as the donor colonies *in situ*, highlighting this as an appropriate method to establish an *ex situ* breeding programme.

Permit restrictions, due to low natural population abundance within Singapore (Bongiorni *et al.*, 2011), severely limited the number of genotypes available for collection. These factors, in addition to concerns over ensuring that the greatest probability of survival during transportation, did not allow for empirical testing of variation in transportation techniques. That said, the method which was utilised achieved 100% post shipment survival, indicating that it is an appropriate method for transporting *A. hyacinthus*. However similar investigations of transportation techniques for other species will be required in the future due to potential variation amongst species in their responses to the method utilised.

8.3 Chapter 3: Inducing broadcast coral spawning *ex situ*: closed system mesocosm design and husbandry protocol

While broadcast coral spawning has occurred within public aquariums (Nosratpour, 2008), such events were unplanned and unpredictable. As a result coral reproductive studies rely on transporting broodstock colonies from natal reefs to holding systems a few days prior to gamete release (Humphrey *et al.*, 2008; Edwards *et al.*, 2010; Chui *et al.*, 2014) or housing corals in open flow-through aquariums exposed to natural sunlight (Lin *et al.*, 2018). Indeed, it has been proposed that inducing broadcast spawning in closed systems is not possible without such environmental cues as photoperiod and lunar cycles (Leal *et al.*, 2014). Therefore, access to gamete material has historically been limited to facilities in close proximity of natal reefs, restricting the number of institutions globally that can study broadcast coral reproduction. Designing a closed system mesocosm, that enabled environmental parameters associated with stimulating broadcast corals to complete gametogenesis and trigger gamete release *ex situ*, aimed to ameliorate this restriction and was a fundamental aspect of this thesis. By mirroring values of seasonal temperature change, photoperiod, seasonal solar irradiation and lunar cycle, five species of broadcast spawning *Acropora* corals were induced to successfully spawn and complete gametogenic cycles, providing material for use in other studies within this thesis. Whilst not the main focus of this thesis, I have now spawned an additional 16 hermaphroditic broadcast coral species (21 species in total) (Fig 8.4), highlighting its broader success for research applications.

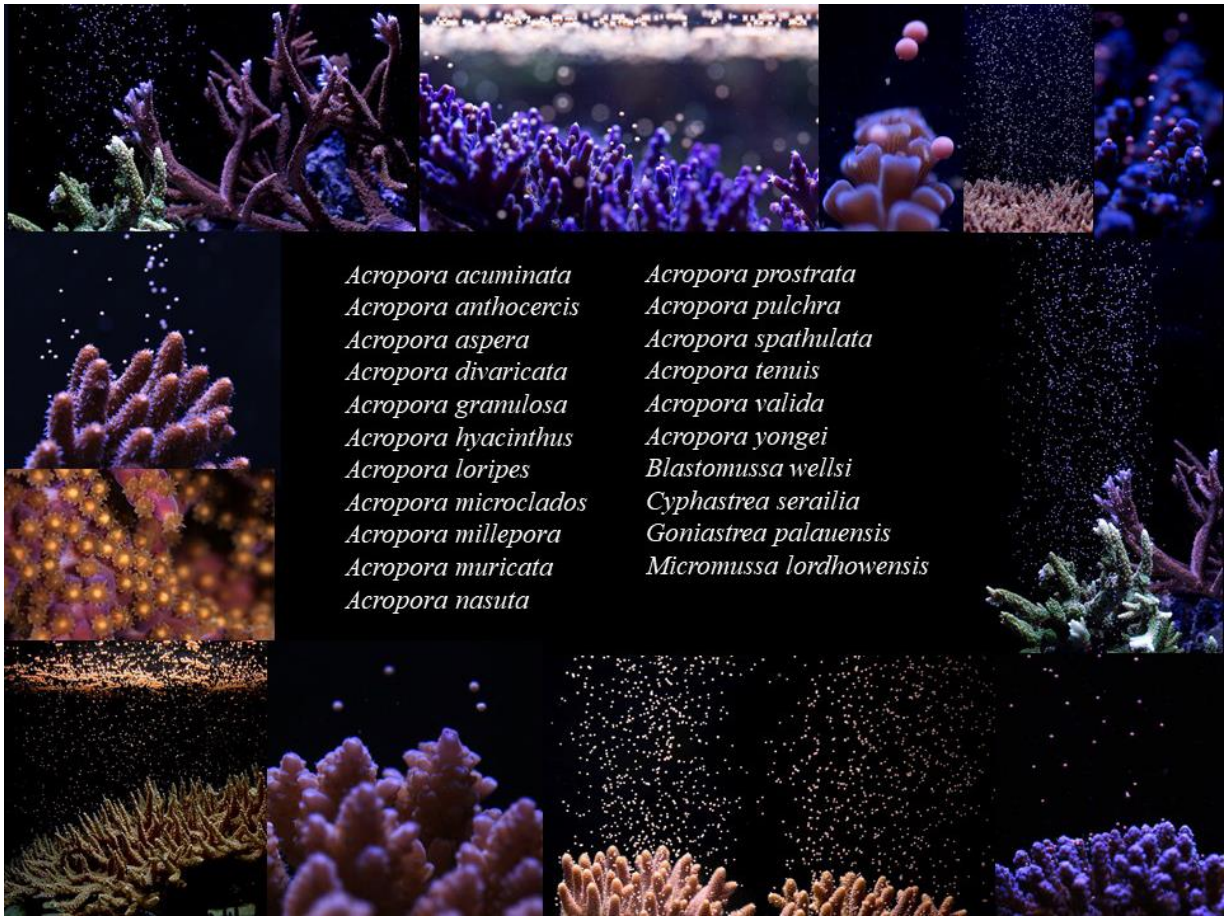


Figure 8. 4 Observed *ex situ* spawning of 21 broadcast coral species at the Horniman Museum and Gardens.

All images taken by J Craggs

8.3.1 Observed delay in ex situ lunar phase spawning

An interesting anomaly associated with inducing *ex situ* spawning within this mesocosm design is the delay in gamete release of approximately 3 to 4 nights compared to *in situ* recorded events (Table 8.1). Synchronicity of spawning night (within the lunar phase) has been shown to be a light mediated response (Gorbunov and Falkowski, 2002; Boch *et al.*, 2011). The period of absolute darkness post sunset prior to moonrise, extends by approximately 47 mins with each passing night following a full moon. Genes within corals are regulated by this spectral dynamic (Kaniewska et al. 2015) and as such, during the re-design of the mesocosm (Chapter 3), great effort was made to try and minimise any confounding effects. Integrated blinds built into the frame eliminated any naturally occurring external light influence. In addition, all LED light sources from pump controllers or heaters were taped over to ensure they did not disrupt this gene regulation and adversely influence the spawning behaviour. The consistent delay in *ex*

situ spawning behaviour therefore must be a result of other influencing factors. This has led me to speculate on the potential role of hormonal cues in triggering spawning events. The steroid estradiol-17ß shows an eightfold increase *in situ* during broadcast spawning events (Atkinson and Atkinson, 1992) and plays an important role in synchronising the growth and development of oocytes within coral. In addition glucuronided estradiol and immunoreactive gonadotropin-releasing hormones are elevated during the spawning period and may trigger spawning via chemical messages in the water column (Twan *et al.*, 2006). Future work should explore these aspects in more detail. Further, the filtration of the mesocosm incorporates protein skimming and activated carbon filtration, designed to remove any build-up of organic compounds from the water. These filtration components may also delay a build-up of a critical threshold of hormones within the water column potentially, in part, explaining the observed delay in all *ex situ* spawning observations to date. The role of hormone dilutions within mesocosms and any influence on *ex situ* spawning behaviour therefore warrants further research.

Table 8. 1 Comparison of broadcast coral spawning behaviour from predicted *in situ* patterns and observed *ex situ*.

	Species	Predicted *in situ* spawning date	Observed *ex situ* spawning date
Chapter 2	*A. hyacinthus*	Singapore 5 NAFM	8-9 NAFM
Chapter 3	*A. hyacinthus*	Singapore 5 NAFM	8-10 NAFM
	A. millepora	GBR 4-7 NAFM	9-16 NAFM
	A. microclados	GBR No comparative data	14 NAFM
	A. tenuis	GBR 3-6 NAFM	6-9 NAFM
Chapter 4	*A. anthocercis*	GBR No comparative data	9 NAFM
	A. millepora	GBR 4-7 NAFM	9 NAFM
	A. tenuis	GBR 3-6 NAFM	7 NAFM
Chapter 5	*A. millepora*	GBR 4-7 NAFM	9 NAFM
	A. anthocercis	GBR No comparative data	9 NAFM
Chapter 6	*A. millepora*	GBR 4-7 NAFM	10 NAFM
Chapter 7	*A. millepora*	GBR 4-7 NAFM	7-8 NAFM

8.3.2 Phase shifting seasonal broadcast spawning events

Spawning of broadcast corals, at least in synchrony with wild populations in fully closed *ex situ* mesocosms, is fundamentally driven by the knowledge of the temporal spawning behaviour of each species at a given location *in situ*. Utilising microprocessor technology to replicate the environmental conditions to stimulate the spawning facilitates this. Access to data from the natal reefs is an important facet and multiple datasets are freely available online:
photoperiod and lunar cycles (www.timeanddate.com), GBR sea surface temperature (data.aims.gov.au/aimsrtds/yearlytrends.xhtml) and
solar irradiance (https://power.larc.nasa.gov/data-access-viewer/).
Gamete release can be largely in synchrony with the wild, but spawning time can be manipulated to occur during the day through controlling the light/dark regime with integrated blackout blinds (Craggs et al. 2017, Chapter 3). This phase shifting of *ex situ* spawning is logistically more convenient, with *Acropora tenuis* spawning between 11.00-11.30 GMT and *Acropora anthocercis, A. hyacinthus, Acropora microclados* and *Acropora millepora* spawning between 13:00 and 16:00 GMT. In addition, the *ex situ* environment offers considerably more control and negates potential confounding effects associated with an *in situ* environment. For example, debate remains about the putative roles that solar irradiation (Penland *et al.*, 2004; van Woesik, Lacharmoise and Köksal, 2006) and seasonal temperature (Keith *et al.*, 2016) play in triggering synchronous spawning. However, disentangling the effects of these environmental variables *in situ* is challenging as they are strongly correlated. *Ex situ* research supports such investigations by providing the ability to vary individual environmental parameters orthogonally through computer control and highlights the possible role this may play in the future.

Such increased control over coral spawning behaviour leads to a logical progression of *ex situ* breaking of the natural spawning cycles to create multiple spawning events within a year. The construction of numerous mesocosms in which the seasonal environmental data set is phase shifted by different amounts could facilitate fairly continuous spawning within a single facility. Such a process could provide year-round access to material for research as well as supporting the scaling up of restoration efforts via the production of coral seed within land based nurseries. While not a main focus of this thesis, initial research conducted between 2015–2018 on phase shifting of all environmental parameters associated with inducing spawning events indicates that such manipulation is not only possible (Table 8.2) but that the same genotype can also be

induced to spawn twice in a single year (AH2, Table 8.2 B & D). In this pilot study *A. hyacinthus* colonies imported from Singapore (Chapter 2) were 'reprogrammed' to GBR data and shifted their spawning cycle to match counterparts of the GBR (Table 8.2 C-E & H). These colonies were subsequently 'reprogrammed' by phase shifting the GBR data set backwards by five months, resulting in the Singaporean genotypes spawning in August 2018 (Table 8.2 G). This highlights the plasticity of the spawning behaviour within the species and the substantial potential of *ex situ* spawning for supporting research and restoration work.

Table 8. 2 Initial data of manipulation of *ex situ Acropora hyacinthus* (Singapore genotypes) spawning behaviour via environmental data phase shifting.

(A) following transportation from Kusu Reef, Singapore, 12 genotypes spawn within the same lunar month as *in situ* colonies; (B) following completion of gametogenic cycle four colonies spawn within the same lunar month as *in situ* colonies; (C) mesocosm is reprogrammed with GBR environmental data July 2016; (D) three genotypes spawn within the GBR spawning 2016 season; (E) three genotypes spawn within the GBR spawning 2017 season; (F) following 2017 spawning all colonies are divided and split between two mesocosms. One based on GBR data (1), the second programmed with GBR data phase shifted for August 2018 spawning (2); (G) six genotypes spawn August 2018 in mesocosm 1; (H) two genotypes spawn in synchrony with GBR.

	Predicted *in situ* spawning	Observed *ex situ* spawning	Number genotypes spawning *ex situ* (genotype codes)
	Mesocosm programmed on Singapore data		
A	7^{th} – 10^{th} April 2015 (3-6 NAFM) Singapore	10^{th} – 13^{th}April 2015 (6-9 NAFM)	n=12 (AH1,2,4,5,6,7,8, 9,10,12,13 &14)
B	26^{th} – 29^{th} March 2016 (3-6 NAFM) Singapore	31^{st} March – 2^{nd} April 2016 (8-10 NAFM)	n=4 (AH 2,7,12 & 13)
C	July 2016 mesocosm reprogrammed with GBR data		
D	26^{th} – 29^{th} March 2016 (3-6 NAFM) Singapore 17^{th} and 21^{st} Nov 2016 (3-7 NAFM) GBR	29^{th} Nov 2016 (15 NAFM) 21^{st} – 22^{nd} Dec 2016 (7-8 NAFM)	n=3 (AH 2,4 & 14)
E	14^{th} – 17^{th} April 2017 (3-6 NAFM) Singapore 7^{th} – 11^{th} Dec 2017 (3-7 NAFM) GBR	13^{th} Nov (9 NAFM) 15^{th} – 16^{th} Dec 2017 (11-12 NAFM)	n=3 (AH 2,3 & 4)
F	10 genotype colonies divided in two. One piece into a mesocosm (1) with GBR data phase shifted for August 2018 spawning. One piece remained in a mesocosm (2) base on normal GBR data		

G	3rd – 6th April 2018 (3-6 NAFM) 26th – 30th Nov 2018 (3-7 NAFM) GBR	Mesocosm 1 5th – 6th August 2018 (8-9NAFM)	n=6 (AH 2,3,4,5,7,13)
H	3rd – 6th April 2018 (3-6 NAFM) 26th – 30th Nov 2018 (3-7 NAFM) GBR	Mesocosm 2 27th Nov 2018 (3 NAFM)	n=2 (AH 7,14)

8.3.3 Impact of mesocosm design and ex situ spawning capability

The logistical convenience of *ex situ* spawning and the potential to phase shift spawning seasons have led a number of institutions to replicate the mesocosm design to meet their own research criteria. Descriptions of those institutions and the applications of the methods are presented below.

8.3.3.1 Center for Conservation (CFC) at Florida Aquarium

Over the past 10 years the husbandry team at CFC have been conducting restoration work along the Florida reef track in partnership with the Coral Restoration Foundation. Initially this work focused on transplantation of asexual fragments but increasingly efforts have focused on the production of sexual propagules to increase genetic diversity of the transplanted population. In the development of scaling these coral conservation efforts, in 2016 the construction of the CFC, a satellite facility of Florida Aquarium located in Apollo Beach, Tampa Bay commenced. This 22 acre site, which is part of a several hundred acre parcel of land called the Florida Conservation and Technology Center, is now the largest land based Caribbean coral archive and research facility currently in existence (Fig 8.5 A). Two greenhouses with high-end specifications, encompassing an area of 3,000 square feet serve as a genetic repository for U.S Endangered Species Act (ESA) listed corals (Fig 8.5 B). These including; *Acropora cervicornis, Acropora palmata, Orbicella faveolata Meandrina meandrites* and *Dendrogyra cylindrus.* All of these have experienced precipitous declines throughout the Caribbean in the past 30 years (Porter and Meier, 1992; Graham *et al.*, 2014). Indeed recent widespread disease along the Florida reef track has resulted in 95% density loss of *Meandrina meandrites* with just five colonies remaining *in situ* in 2016 (Walton, Hayes and Gilliam, 2018).

These two greenhouses also act as a grow out facility for sexually produced spat, currently from *in situ* gamete collection. Over the past two years (2017 & 2018) over 3000 genetically diverse colonies have been grown on (Fig 8.6) for translocation into the field. In the future these greenhouses will facilitate the grow-out of *ex situ* produced offspring. To this end an additional research building, covering 15,000 square feet, was completed in 2018, housing labs, offices, and climate controlled research space for intensive highly controllable coral culture (Fig 8.5 B). Within this building, four replicate mesocosms (Fig 8.5 C) have been built, housed within two climate controlled rooms. The immediate objectives of these four mesocosms will be for one system to accommodate *Acropora cervicornis*, two systems to house *Dendrogyra cylindrus* and the fourth system will house disease-free *Meandrina meandrites,* (a species for which very little is known about its reproduction). Longer term goals include seasonal phase shifting to induce multiple spawning events per year, increasing productivity in order to support greater reef transplantation.

This *ex situ* land based seed production model is akin to tropical reforestation practises in which tree seedlings are often grown in nurseries prior to transplantation to targeted sites (Evans, 1992). Indeed similar challenges of scale, cost of direct seeding versus planting nursery raised seedlings, seedling survival and species selection that ensure appropriate community structure within a restored population (Stimm *et al.*, 2008; Cole *et al.*, 2011) present themselves in both forest and coral systems. Reforestation practises are well established, as are long-term effectiveness evaluations of projects, that take into account socio-economic, biophysical and project objectives (Le *et al.*, 2012). With such strong parallels, important lessons learnt, from the more established terrestrial models, may also prove to be highly relevant for reef restoration practises in the future.

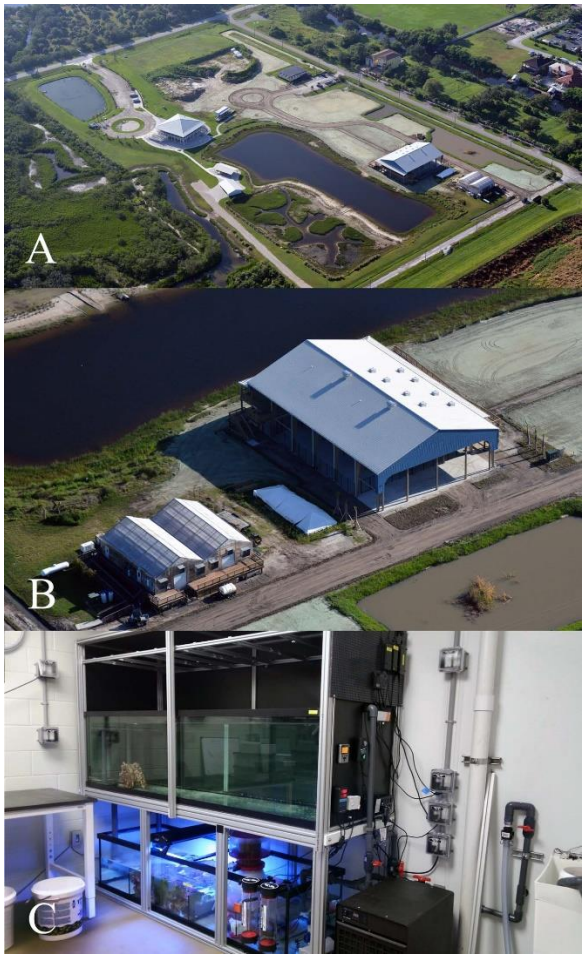


Figure 8. 5 Center for Conservation, Tampa Bay, Florida USA.
(A) Aerial image of the 22 acre site of Center for Conservation; (B) Two greenhouses housing a genetic repository of U.S Endangered Species Act listed corals from the Florida reef track. A research building housing labs, offices, and four replicate mesocosms for *ex situ* Caribbean broadcast spawning; (C) One of four mesocosms at CFC replicating the mesocosm design at the Horniman Museum and Gardens. Photograph credit: A & B ©Aero Photo, C © Keri O'Neil, CFC.

Figure 8. 6 Caribbean hard coral species growing out at Center for Conservation, Florida, following *in situ* gamete collection.

(A) 9-month-old *Orbicella faveolata* colonies; (B) 9-month-old *Acropora palmata* colonies; (C) 18-month-old *Acropora cervicornis* colonies; (D) 6-month-old *Dendrogyra cylindrus* polyp. *Photograph credit; C & D* © Keri O'Neil, CFC. *Other images taken by J Craggs*

8.3.3.2 California Academy of Science, Steinhart Aquarium

Following consultations with the California Academy of Science, in 2018 a six tank system (Fig 8.7 A & B) that emulates the full seasonality programming and filtration developed in chapter 3 was constructed. This system emulates environmental conditions in Palau and in February 2018 broodstock colony fragments of the broadcast spawning species *A. hyacinthus*, *A. tenuis* and brooding corals *Stylophora pistillata* and *Pocillopora damicornis* were transported to the USA, following methods described in chapter 2. Multiple *A. hyacinthus* colony spawning occurred *ex situ* on 16th April 2018, 16 NAFM (Fig 8.7 C), the first planned *ex situ* spawning in the US and resulted in the production of F1 embryos via successful *in vitro* fertilisation. Future aims for the research focus on understanding effect of ocean acidification on spawning events.

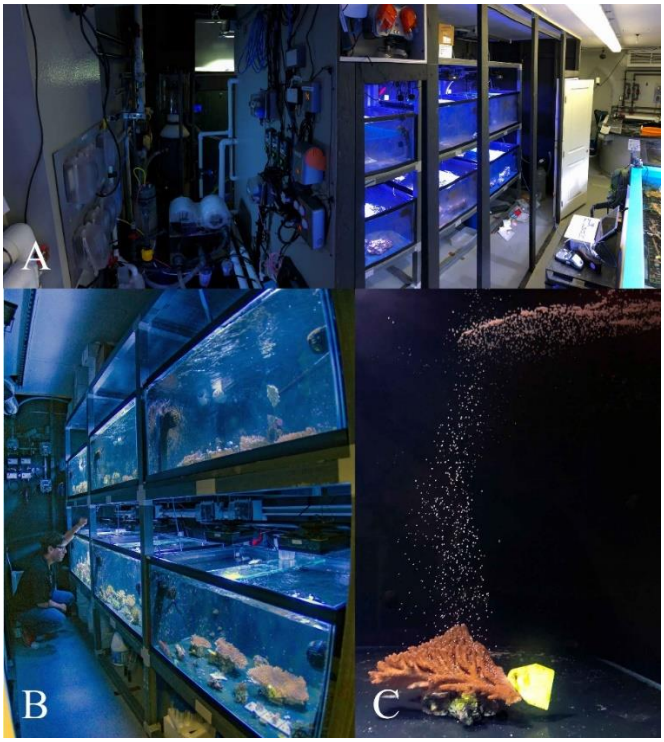


Figure 8. 7 *Ex situ* closed system mesocosm at California Academy of Science based on system design in chapter 3.

(A) Six tank mesocosm design with microprocessor, mechanical filtration and external framework for black out boarding; (B) Gravid broodstock *Acropora hyacinthus* colonies from Palau within the holding tanks shortly after transportation; (C) *A. hyacinthus* spawning *ex situ* on 16th April 2018, 16 NAFM. *Photograph credit; A-C* © Richard Ross, California Academy of Science.

8.3.3.3 Kenkel's Lab, University of Southern California, Dornsife

Research at the Kenkel lab focuses on local adaptation, biomarker development and evolutionary ecology in Cnidarians. Two 2000 litre systems are used to hold broodstock coral colonies (experimental and holding), with an additional modular system with 16 tanks, that can either be run independently or collectively with environmental regimes where temperature, flow rate, lighting regime and water pH can be controlled. The lab aims to focus on the brooding and broadcast spawning of corals *ex situ*, with methods to achieve this being based on protocols developed in this thesis. To this end average yearly onshore and offshore Florida Keys temperature profiles have been added to the two broodstock systems (experimental and holding) using the microprocessors configurations and code described in chapter 3 (Craggs *et al.*, 2017).

8.4 Chapter 4: Embryogenesis of reef building corals following *ex situ* induced gametogenic cycles and subsequent broadcast spawning events

The platform of planned *ex situ* spawning within closed system mesocosms offers considerable potential for coral population management within aquarium collections (Petersen *et al.*, 2006; Petersen, 2008), for coral reproductive research (Chui *et al.*, 2014) and supporting reef restoration through the provisioning of sexual recruits (Edwards *et al.*, 2010). However this new approach is in its infancy and as such there is a dearth of research on any consequences, positive or negative, for embryological development as a result of gametogenic cycles being completed *ex situ*. To date, only one study (Lin *et al.*, 2018) has investigated gamete development in aquarium housed broadcast spawning species but these individuals were housed in open seawater tanks exposed to natural photoperiod and lunar cycles. The aim of this chapter was therefore to document embryogenesis from broodstock colonies that have completed gametogenesis *ex situ* rather than from recently collected from the wild.

Empirical data on egg sperm bundle ascending speeds and bundle dissociation times showed significant differences between species, possibly providing broader insights into reproductive adaptions to environmental zonation (Arai *et al.*, 1993) and the implication of pre-zygotic barriers (Wei et al. 2012; Numata and Helm 2014) that drive evolutionary processes (Willis *et al.*, 2006).

Scanning electron microscopy revealed that oocytes were smaller in species that completed gametogenesis *ex situ* than those previously reported *in situ.* Such differences may indicate a confounding factor of a captive environment and could be a consequence of *ex situ* broodstock nutrition, a factor that has previously been shown to influence gamete quality, determined in maricultured marine invertebrates (Berntsson *et al.*, 1997; Carboni *et al.*, 2015).

Despite this smaller size, fertilisation rates were high in all species, and comparable to *in situ* spawning. In addition no abnormal development of embryos was detected, implying the absence of negative effects of *ex situ* spawning. However, further investigations to determine the quality of embryos, such as embryo respiration (Lopes, Greve and Callesen, 2007; Okubo *et al.*, 2008), as well as potential implication for gamete production of *ex situ* coral nutrition (water chemistry effects on autotrophic sources of fixed carbon source and heterotrophic inputs) are warranted.

8.5 Chapter 5: Interspecific hybridisation and multi genotype aggregations enhance survivorship during early ontogeny in a reef building coral

Scleractinian corals undergo a bottleneck in survival post settlement (Guest *et al.*, 2014) and understanding factors that drive mortality during early ontogeny may generate ideas on how to alleviate this pressure and increase survival rates. In chapter 5, I explored how the effects of the genetic crosses (pure vs interspecific hybridisation) and the number of genotypes within post settled entities (1, 2, 3 & >3 genotypes) on survivorship and growth. Results indicate that multi-genotype entities with more than two genotypes, in all genetic crosses, result in a survival benefit over those settling singly. In addition, interspecific hybridisation improved survivorship compared to the pure genetic cross of *A.millepora* / *A.millepora*. However, factors of allorecognition maturity and the degree of relatedness within a settled cohort (not tested in this study) may have influenced the results. When developing methods to maximise output productivity of sexual recruits for research and restoration, important consideration is needed of the allorecognition maturity, which develops earlier in non-related multi-genotype entities, drives genotype rejection responses and results in higher mortality (Puill-Stephan *et al.*, 2012a, 2012b). Production of fully related cohorts, via appropriate *in vitro* fertilisation methods, would likely circumvent such confounding effects and maximise productivity of corals generated for restoration practises.

Although experimental conditions were highly controlled, the local environmental conditions that corals experienced post settlement were very stochastic, with factors outside of experimental control influencing survival. In particular, negative interactions with benthic algae species were observed in all genetic cross and entity types. This was often observed as a diatom or cyanobacterial growth around the peripheral edge of the colonies. Complex coral-algae interactions have been shown to induce disease and coral mortality (Vermeij *et al.*, 2009). Indeed, macro and turf algae promote microbial overgrowth of coral and also induce mortality via direct physical harm from hydrophobic organic matter (Barott and Rohwer, 2012). Observing these interactions within this experiment led to the development of the concept of utilising microherbivory in a co-culturing approach to control algae growth and therefore enhancing coral survival (Chapter 6).

8.6 Chapter 6: *Ex situ* co culturing of the sea urchin, *Mespilia globulus* and the coral *Acropora millepora* enhances early post-settlement survivorship: implications for large scale propagation

As reef systems decline amelioration work to restore local coral populations is becoming increasingly important (Omori, 2011; Ng, Toh and Chou, 2016). As a result new approaches such as assisted gene flow (van Oppen *et al.*, 2014), hybridisation (Chan *et al.*, 2018) and mass production of seed units (Chamberland *et al.*, 2017) are being developed to support such goals. Methods that improve productivity must therefore be a core focus if such initiatives are to be effective at scales that are impactful ecologically. Post-settlement mortality is driven by multiple complex benthic interactions including predation, environmental stresses and, importantly, benthic competition with algal species. When considering management of post settlement recruits for restoration, the labour required to clean settlement tiles remains a high cost and the need to minimise this cost provided the impetus for investigating the concept of co-culturing and the utilisation of microherbivory for improved coral survival (chapter 6). Co-culturing broadcast corals (*Acropora millepora*) with juvenile sea urchins (*M. globulus*) provided some very encouraging results. Not only did coral survivorship after 6 months increase from 5% in the negative control (with no grazing), to just under 40% in the highly grazed treatments, but there was also the increased benefit of colonies growing faster. The results of this experiment highlight the substantial potential that co-culturing could play if applied to restoration settings. It is important to note that such an approach of dual species rearing does increase husbandry challenges. Also, there are significant logistic considerations in coordinating timelines of urchin spawning and coral gamete release in order to allow sufficient time for the urchin pelagic larval phase, settlement and grow out to be completed prior to coral spawning and settlement. Further, the experiment was conducted on a small scale. Independent, mass production of coral (Pollock *et al.*, 2017) and urchins (Westbrook *et al.*, 2015) is possible. A future research focus should therefore be to assess the feasibility of tandem production at a scale that promotes ecological *in situ* benefits.

Combining the benefits of microherbivory to promote coral production may drive down the cost per unit of coral for restoration. Additionally opportunities may also present themselves if the production of the co-culturing species could provide sustainable livelihood and mariculture options for human food markets. In this regard the collector sea urchin, *Tripneustes gratilla* would be an ideal co-culturing species in Indo-Pacific regions. Such an approach may provide

further cost reductions of individual coral units for restoration by improving productivity, whilst also providing an income source for coastal communities that apply these techniques. The potential for microherbivory to contribute to reef restoration and sustainable livelihood development therefore warrant future investigation.

8.7 Chapter 7: Closing the life-cycle

Annually, more than 700 million people visit zoos and aquariums and collectively these institutions raise an estimated US$350million for wildlife conservation (Gusset and Dick, 2011). As such, these institutions and the living collections they hold, are important conduits to educate the public about the natural world. Additionally they have long played a role in both *in situ* (Gusset and Dick, 2010) and *ex situ* (Conde *et al.*, 2011; Zippel *et al.*, 2011) species conservation, which is regarded as an intrinsic characteristic of managing a living collection. The clear trend of increased species extinction as a result of anthropogenic influences (Dirzo *et al.*, 2014) highlights the increasingly important role that zoos and aquariums must play in species conservation and it has been suggested that greater emphasis must be placed on *ex situ* programmes (Pritchard *et al.*, 2012). Indeed, recent works highlight the number of endangered and critically endangered fish and coral species that are held within zoo and aquarium collections around the world (da Silva *et al.*, 2019), providing an important focus for *ex situ* programme species priorities. Long term success is determined by closing the life cycle in order to diversify the gene pool of a collection, support stock management and exchanges between collections and reduce the collection of individuals in the wild (Wilson and Vincent, 1998).

The development of an *ex situ* broadcast coral spawning programme would support such species conservation aims. The production of multiple filial generations (F1 & F2) of *Acropora millepora* in a closed system mesocosm described in chapter 7 marks the first successful closing of the life-cycle of broadcast spawning corals *ex situ*. However, it should be noted that the numbers that spawned were low (n=2). Further, only six colonies survived to adulthood, after a three year period and therefore a great deal of improvement is required, particularly in long-term husbandry. Mistakes in water chemistry management during the three years resulted in colony deaths which may reflect staff capacity and resources to appropriately manage the collection.

That said, the achievement of closing the life cycle provides a platform to explore new opportunities. The distribution of *ex situ* reared genotypes between aquarium collections could support sustainable stock management alleviating pressure on wild population (Petersen *et al.*, 2007). In addition, the effectiveness of introducing *ex situ* reared livestock to the wild has rarely been tested, but in some taxa can be both economical and biologically effective (Santos *et al.*, 2009). Such a goal of *ex situ* breeding programmes supporting *in situ* reef restoration must be an aspiration for the future.

8.8 Conclusion

The overall aim of this thesis was to develop a method to induce broadcast coral spawning in an *ex situ* environment (Fig 8.8 A) and then utilise this as a platform to investigate methods for increasing post-settlement survival by understanding the pressures that drive early post settlement mortality. Sexually mature broodstock colonies were successfully transported from Singapore to the United Kingdom, establishing a captive breeding cohort (Fig 8.8 B). Central to the thesis was designing a mesocosm (Fig 8.8 C) that allowed full gametogenic cycles of *Acropora* to be completed and, for the first time, planned and predictable *ex situ* gamete release to occur within the same lunar month as wild counterparts.

The first two chapters created methods that meant other institutions (away from tropical reef locations), can now, establish their own sexual reproductive research programmes. This output will considerably increase the breadth of research practices, whether these be associated with understanding the impacts of climate change on coral reproduction or the development of new techniques to upscale reef restoration practices. The use of these practises therefore marks an important applied output to the work described herein.

The slightly smaller size of oocytes produced from species completing full gametogenic cycles *ex situ* warrants further research, particularly the role of broodstock nutrition (autotrophic and heterotrophic) on maternal resource provisioning (Fig 8.8 D), along with any implications, (positive or negative), to post settlement survival.

When considering the application of *ex situ* spawning, either as a direct source of sexual recruits for reef restoration practices or as the basis to conduct experimental work focusing on developing methods to increase productivity for these initiatives, a number of key considerations have presented themselves throughout this thesis. Interspecific hybridisation vigour, in part, appears to have increased survival compared to some pure species crosses (Fig 8.8 E). However, the allorecognition maturation period, as a consequence of cohort relatedness, must be considered. Whilst conducting pure pair-wise crosses during *in-vitro* fertilisation increases the logistical workload during spawning the positive impact of increasing survival for the first 12 weeks in early post-settlement recruits (through delaying the allorecognition system in the resulting fully related offspring), might be an important factor in improving productivity for reef restoration.

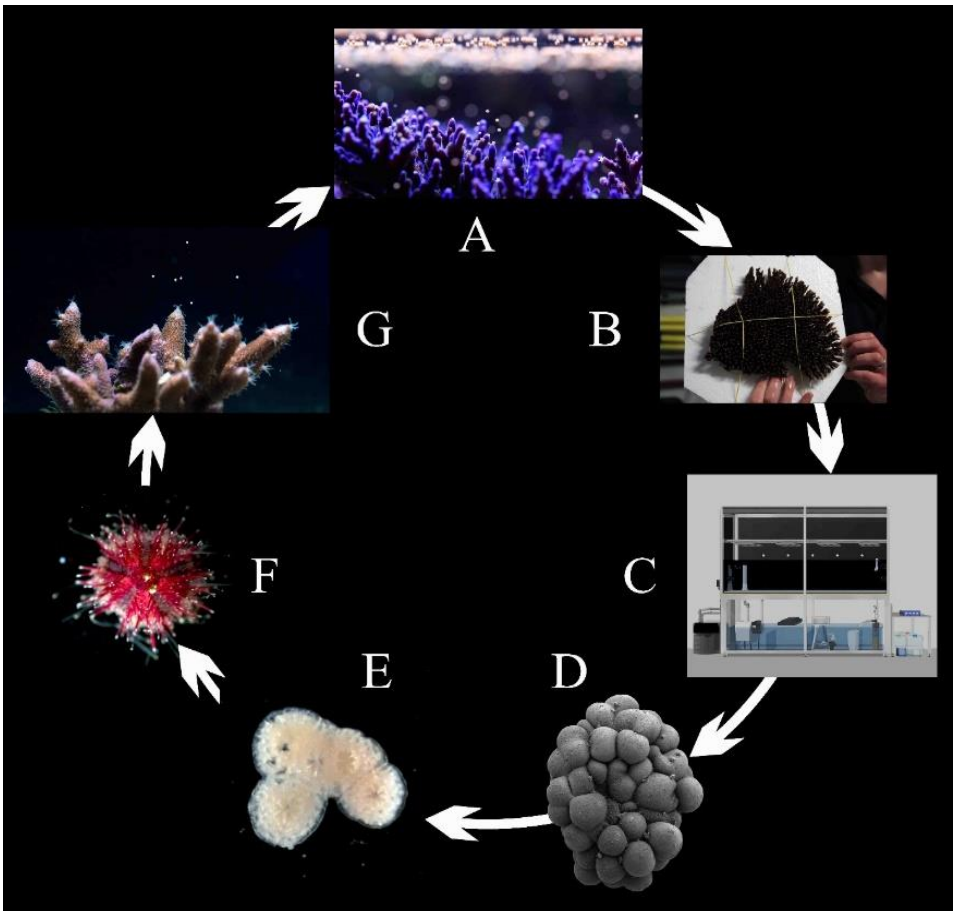


Figure 8. 8 Overview of thesis achievements.
(A) the first induced *ex situ* broadcast spawning *A. valida*; (B) successful long distance transportation of gravid *Acropora hyacinthus* broodstock colonies to establish a breeding group; (C) mesocosm design that can replicate environmental parameters (seasonal temperature, photoperiod, solar irradiance and lunar cycle) associated with broadcast spawning; (D) normal embryological development occurs from coral that complete gametogenic cycles *ex situ*; (E) multi genotype entity show improved survival over single genotype entities in both pure and interspecific hybridised genetic crosses; (F) co-culturing utilising microherbivory enhances post settlement coral survival; (F) closing the life cycle of *Acropora millepora ex situ* in 3 years. *All images taken by J Craggs*

Post settlement survival rates in chapter 5 were low, potentially as a consequence of interactions with algae. However, the role heterotrophic feeding has with regard to post-settlement survival is well documented (Petersen, Wietheger and Laterveer, 2008; Conlan *et*

al., 2017). This is because, Symbiodiniaceae uptake only occurs from 7 days post metamorphosis in horizontal transmission species. Therefore, the application of different food sources (at this early stage – before the acquisition of symbiotic algae) should be explored.

Competition with benthic algae is a significant cause of early post settlement mortality. I show that the application of urchin microherbivory (Fig 8.8 F) can play a positive role in the control of these negative interactions with a resulting increase in survival. A density of $75m^{-2}$ juvenile *M. globulus* resulted in the highest overall coral survival, as well as increasing coral growth compared to the non-grazed control. The application of co-culturing for restoration practises should therefore consider this practise and utilise native urchins to assist in coral survivorship within their tank system.

In summary, a combination of the following factors; pair-wise genetic crosses, heterotrophic feeding of early post-settlement spat and co-culturing utilising microherbivory should be incorporated into reef restoration practises and efforts to up-scale the positive ecological impact of these initiatives. Additionally, these approaches must also be incorporated into the *ex situ* management of living collections as a means of generating sustainable sources of reef building corals for the aquarium trade. *Ex situ* breeding programmes offer an additional method to support conservation practises and closing the life cycle of *A. millepora* (Fig 8.8 G), which was described here for the first time, is an important step forward. It must be noted however, that as such practises become more accessible to the broader public aquarium community appropriate management of the genetic structure of captive populations will be needed in order to avoid effects of inbreeding depression over time. Therefore, more work into genotype specific management and an understanding of geographical provenance of founding stocks should be undertaken.

Finally, preliminary data on breaking natural reproductive cycles through environmental phase shifting suggest the possibility of increasing the number of broadcast spawnings per year. The ability to generate multiple reproductive events within a year will dramatically increase the access to material and therefore researcher capabilities to answer questions associated with up-scaling restoration. Such control over coral reproduction *ex situ* is a powerful new contribution to the reef restoration toolbox.

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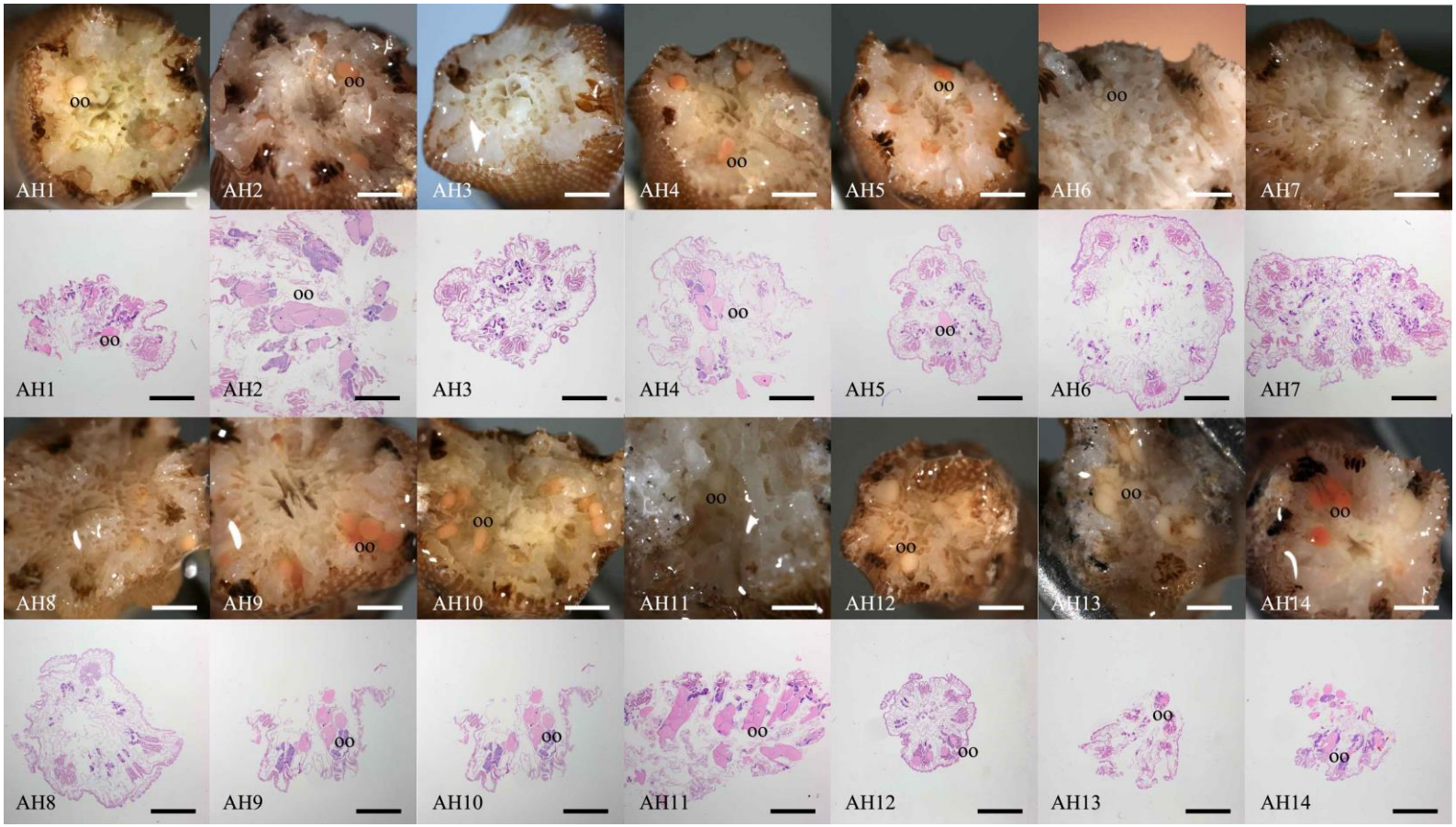
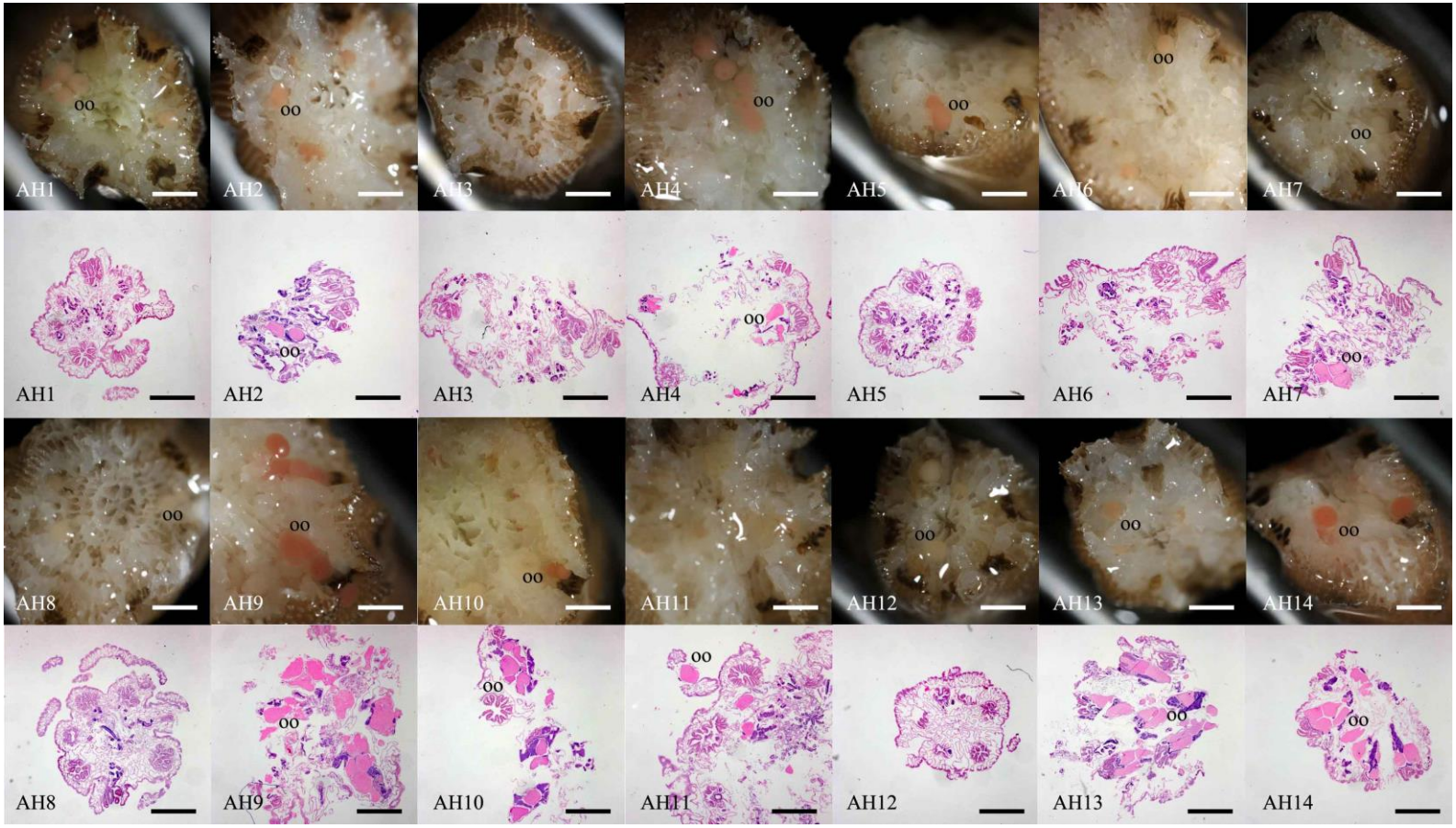
Appendices



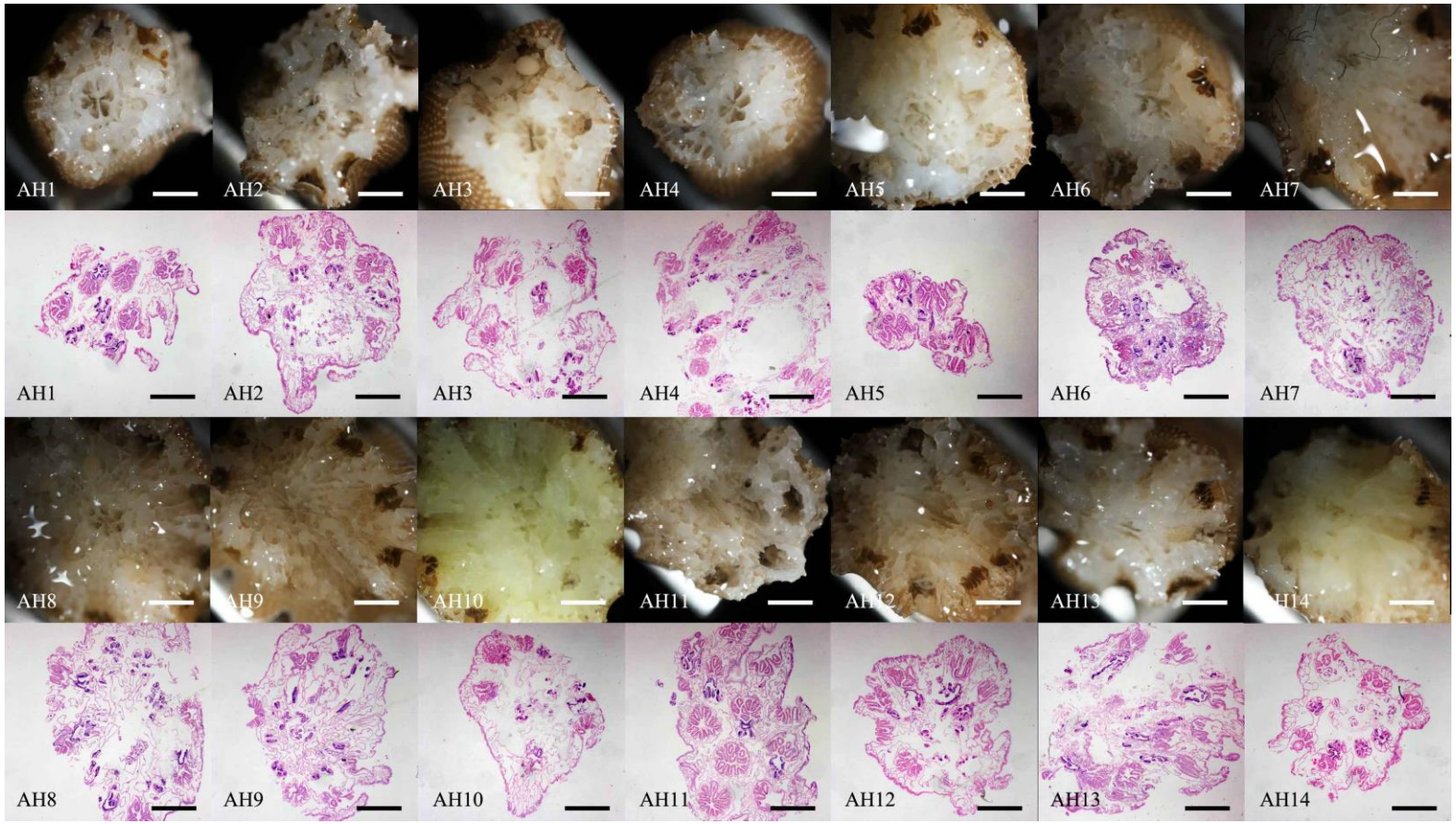
Appendix 1 Cross section of *Acropora hyacinthus* branch fragments and H&E stained histological preparation of colonies AH1-14 on 25th March 2015.

AH1-2, 4-6 8-14 contain pigmented or partially pigmented oocytes (oo). Scale 1mm. *All images taken by J Craggs*



Appendix 2 Cross section of *Acropora hyacinthus* branch fragments and H&E stained histological preparation of colonies AH1-14 on 4th April 2015.

Colonies AH1-2, 4-14 contain mature pigmented oocytes (oo). Scale 1mm. *All images taken by J Craggs*



Appendix 3 Cross section of *Acropora hyacinthus* branch fragments and H&E stained histological preparation of colonies AH1-14 on 23rd April 2015.

Oocytes are absent in all colonies following previous *ex situ* gamete release. Scale 1mm. *All images taken by J Craggs*

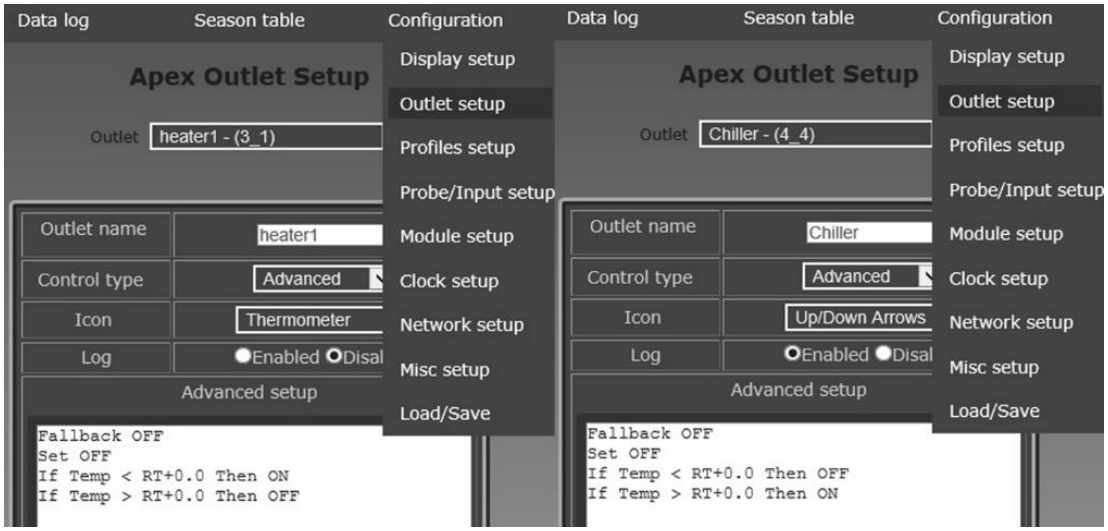
Appendix 4 ICP water test results from Kusu Reef, Singapore and Singapore mesocosm.

		Macro Elements (mg/l)											Li Group (µg/l)			I Group (µg/l)				Fe-Group (µg/l)						Nutrient Group	
		Al	Pb	Cu	Na	Ca	Mg	K	Br	B	Sr	S	Li	Ni	Mo	V	Zn	Mn	I	Cr	Co	Fe	Ba (µg/l)	Be	Si (µg/l)	P (µg/l)	PO4 (mg/l)
Kusu reef Singapore	17/3/15	0.00	0.00	0.00	10220	400.50	1278	378.80	69.01	4.51	7.36	950.0	172.30	0.00	9.99	1.53	0.00	0.00	68.67	0.00	0.00	0.00	6.72	101.90	101.90	3.45	0.011
	Mean	0.00	0.00	0.00	10220	400.50	1278	378.80	69.01	4.51	7.36	950.0	172.30	0.00	9.99	1.53	0.00	0.00	68.67	0.00	0.00	0.00	6.72	101.90	101.90	3.45	0.011
	Median	0.00	0.00	0.00	10220	400.50	1278	378.80	69.01	4.51	7.36	950.0	172.30	0.00	9.99	1.53	0.00	0.00	68.67	0.00	0.00	0.00	6.72	101.90	101.90	3.45	0.011
Singapore mesocosm	27/02/15	0.00	0.00	0.00	10563	420.30	1365	434.40	55.63	4.15	5.22	942.0	220.50	1.34	17.74	0.00	2.14	0.00	19.46	0.00	0.00	0.00	8.43	0.00	21.66	0.00	0.000
	31/03/15	5.67	0.00	0.00	9703	431.90	1292	395.00	60.75	4.09	4.57	1003.0	200.40	0.00	16.48	0.00	2.82	0.00	26.95	0.00	0.00	0.00	6.64	0.38	47.19	1.72	0.005
	20/04/15	0.00	2.70	4.10	9928	470.80	1274	404.70	48.61	4.01	3.50	798.3	195.80	0.00	11.85	0.00	10.28	0.00	18.48	0.00	0.00	0.00	6.18	0.00	32.89	1.86	0.006
	19/06/15	3.70	0.00	3.49	10433	435.60	1296	419.90	50.32	3.80	2.17	926.6	214.40	0.00	14.76	0.00	9.18	0.00	63.65	0.00	0.00	0.00	5.68	0.00	42.98	2.90	0.009
	22/06/15	9.41	0.00	0.00	9951	464.80	1304	403.00	52.96	3.68	5.89	931.9	186.40	0.90	13.98	0.00	1.70	0.00	55.35	0.00	0.00	0.00	2.90	0.00	50.75	2.53	0.008
	07/07/15	7.45	0.00	0.00	9819	467.70	1285	400.40	55.48	4.54	6.20	943.0	187.40	0.00	14.13	0.00	7.01	0.00	55.32	0.00	0.00	0.00	4.11	0.00	78.36	5.28	0.016
	03/08/15	0.97	0.00	0.00	10685	388.10	1356	415.90	49.39	4.37	4.29	970.7	176.60	0.00	9.78	0.00	0.00	0.00	86.05	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.000
	11/09/15	0.00	0.00	0.00	9949	357.00	1317	387.00	52.67	4.26	4.18	834.9	16..40	0.00	11.26	0.00	0.98	0.00	100.50	0.00	0.00	0.00	3.20	0.00	71.47	0.00	0.000
	06/10/15	0.00	0.00	0.00	9794	408.50	1310	388.30	43.84	4.05	4.31	900.6	159.60	0.00	10.81	0.00	0.00	0.00	80.44	0.00	0.00	0.00	1.73	0.00	108.90	2.85	0.001
	13/11/15	0.00	0.00	0.00	10304	515.40	1332	395.00	42.74	4.34	5.49	965.7	147.30	0.00	7.13	0.00	3.78	0.00	56.02	0.00	0.00	0.00	2.37	0.00	194.80	0.00	0.000
	16/12/15	2.37	0.00	0.00	10580	497.10	1367	402.40	55.30	4.20	4.92	967.4	145.80	0.00	6.52	0.00	1.67	0.00	94.64	0.00	0.00	0.00	2.75	0.00	110.30	2.57	0.001
	22/01/16	4.79	0.00	0.00	10248	437.30	1322	383.60	55.76	4.00	6.83	937.1	129.30	2.12	4.04	0.00	0.00	0.00	33.69	0.00	0.00	0.00	1.95	0.00	79.57	2.90	0.001
	19/02/16	3.50	0.00	0.00	9807	502.30	1357	390.40	51.74	4.20	6.21	944.5	11.90	0.00	9.89	2.96	3.27	0.00	77.32	0.00	0.00	0.00	0.00	0.00	60.22	5.07	0.016

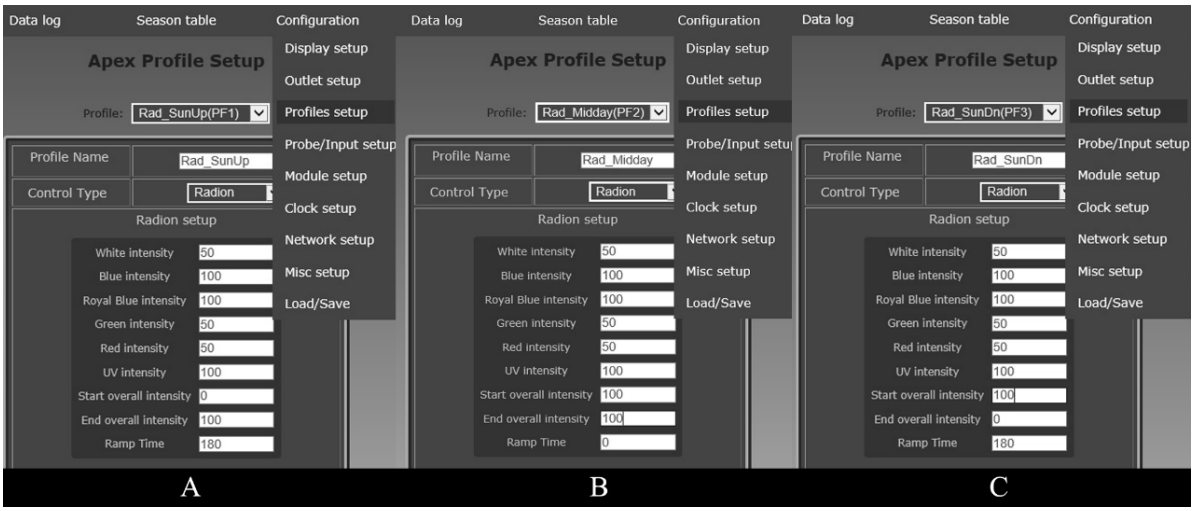
	21/03/16	5.21	0.00	0.00	9567	480.40	1283	365.90	51.00	3.77	4.06	902.5	99.39	0.00	8.37	0.00	1.02	0.00	134.80	0.00	0.00	0.00	1.23	0.00	91.49	11.58	0.035
	27/04/16	2.82	0.00	0.00	10035	462.00	1284	408.00	59.11	4.83	6.96	845.0	144.00	2.51	9.80	0.00	0.00	0.00	99.47	0.00	0.00	0.00	8.13	0.00	50.87	3.72	0.011
	Mean	3.06	0.18	0.51	10091	449.28	1316	399.59	52.35	4.15	4.99	920.9	147.92	0.46	11.10	0.20	2.92	0.00	66.81	0.00	0.00	0.00	3.74	0.03	69.43	2.87	0.007
	Median	2.82	0.00	0.00	9951	462.00	1310	400.40	52.67	4.15	4.92	937.1	168.10	0.00	10.81	0.00	1.70	0.00	63.65	0.00	0.00	0.00	2.90	0.00	60.22	2.57	0.005

Appendix 5 ICP water test results from Lizard Island and GBR mesocosm.

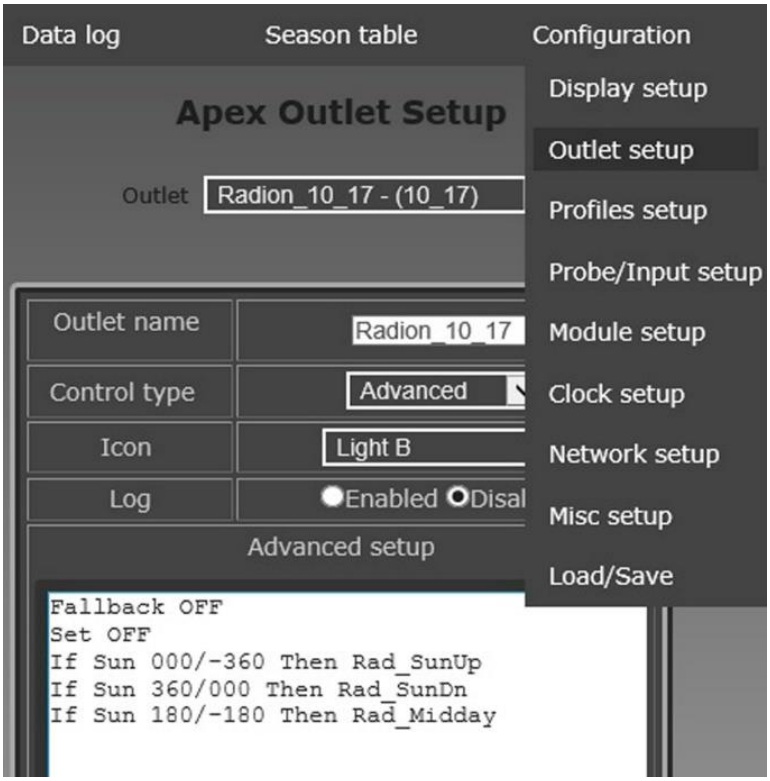
					Macro Elements (mg/l)								Li Group (µg/l)			I Group (µg/l)				Fe-Group (µg/l)						Nutrient Group	
		Al	Pb	Cu	Na	Ca	Mg	K	Br	B	Sr	S	Li	Ni	Mo	V	Zn	Mn	I	Cr	Co	Fe	Ba (µg/l)	Be	Si (µg/l)	P (µg/l)	PO4 (mg/l)
Lizard Island GBR	26/11/15	0.00	0.00	0.00	10530	409.10	1302	384.00	50.36	4.36	8.49	909.1	224.70	0.00	15.75	2.38	0.00	0.00	39.22	0.00	0.00	0.00	7.57	0.00	0.00	5.37	0.016
	27/11/16	0.95	0.00	0.00	11152	475.00	1334	421.00	42.90	4.42	7.19	828.0	216.00	0.00	8.85	0.00	0.00	0.00	30.37	0.00	0.00	0.00	4.18	0.00	21.67	3.31	0.010
	Mean	0.48	0.00	0.00	10841	442.05	1318	402.50	46.63	4.39	7.84	868.6	220.35	0.00	12.30	1.19	0.00	0.00	34.80	0.00	0.00	0.00	5.88	0.00	10.84	4.34	0.013
	Median	0.48	0.00	0.00	10841	442.05	1318	402.50	46.63	4.39	7.84	868.6	220.35	0.00	12.30	1.19	0.00	0.00	34.80	0.00	0.00	0.00	5.88	0.00	10.84	4.34	0.013
GBR mesocosm	06/10/15	3.64	0.00	3.64	10894	449.90	1441	414.10	52.78	4.79	8.52	902.8	225.70	0.00	10.44	0.00	30.64	0.00	14.94	0.00	0.00	0.00	7.11	0.00	67.39	2.01	0.006
	13/11/15	5.94	0.00	8.62	10344	433.70	1386	384.10	47.90	4.32	4.10	927.1	202.80	4.75	7.33	0.00	40.35	0.00	11.54	0.00	0.00	0.00	2.92	0.00	171.10	0.00	0.000
	16/12/15	13.62	0.00	13.38	11001	435.30	1380	407.20	58.19	3.77	4.56	936.1	218.40	4.97	7.96	0.00	37.24	0.00	25.07	0.00	0.00	0.00	5.55	0.00	120.40	2.09	0.006
	22/01/16	7.33	0.00	7.21	10763	421.80	1375	401.40	59.56	3.74	5.85	952.1	202.60	4.49	6.68	0.00	11.03	0.00	20.56	0.00	0.00	0.00	3.63	0.00	129.40	3.40	0.010
	21/03/16	10.90	0.00	4.32	9913	352.50	1293	366.00	53.01	3.39	1.95	900.1	155.60	1.64	7.15	0.00	5.28	0.00	14.27	0.00	0.00	0.00	0.80	0.00	160.70	5.38	0.016
	27/04/16	5.84	0.00	3.49	10267	375.00	1330	408.00	60.85	4.44	3.73	906.0	171.00	2.63	10.01	0.00	0.00	0.00	42.53	0.00	0.00	0.00	2.90	0.00	175.00	2.83	0.009
	11/07/16	1.67	0.00	0.00	10619	441.00	1448	419.00	54.84	4.90	4.05	998.0	159.00	0.00	10.25	0.00	0.00	0.00	88.33	0.00	0.00	0.00	0.00	0.00	206.00	6.78	0.021
	20/08/16	2.36	0.00	0.00	10875	431.00	1457	438.00	57.00	4.83	6.53	936.0	180.00	0.00	7.81	0.00	2.03	0.00	96.00	0.00	0.00	0.00	3.34	0.00	155.00	2.69	0.008
	08/12/16	4.76	0.00	0.00	10477	507.00	1446	434.00	56.18	3.92	3.75	945.0	184.00	0.00	5.56	0.00	0.00	0.00	30.23	0.00	0.00	0.00	4.87	0.00	454.00	6.11	0.019
	21/01/17	11.00	0.00	0.00	10629	502.00	1430	425.00	41.00	4.50	4.20	1026.0	171.00	0.00	4.97	0.00	0.00	0.00	30.00	0.00	0.00	0.00	10.00	0.00	431.00	17.00	0.052
	Mean	6.706	0	4.066	10578	434.92	1399	409.68	54.131	4.26	4.724	942.9	187.01	1.848	7.816	0	12.657	0	37.347	0	0	0	4.112	0	206.999	4.829	0.0147
	Median	5.89	0.00	3.57	10624	434.50	1408	411.05	55.51	4.38	4.15	936.1	182.00	0.82	7.57	0.00	3.66	0.00	27.54	0.00	0.00	0.00	3.49	0.00	165.90	3.12	0.01



Appendix 6 Code for heater and chiller to replicate the seasonal temperature change. Programmed via the Apex classic dashboard, drop down menu configuration, outlet setup.



Appendix 7 Photoperiod profile settings programmed via Apex classic dashboard. Drop down menu configuration, profiles. Radion XR30w Pro LED channels (White, Blue, Royal Blue, Green, Red and UV) set to 50, 100, 100, 50, 50, 100% respectively, to replicate (A) daily sunrise; (B) midday sun; (C) sunset.

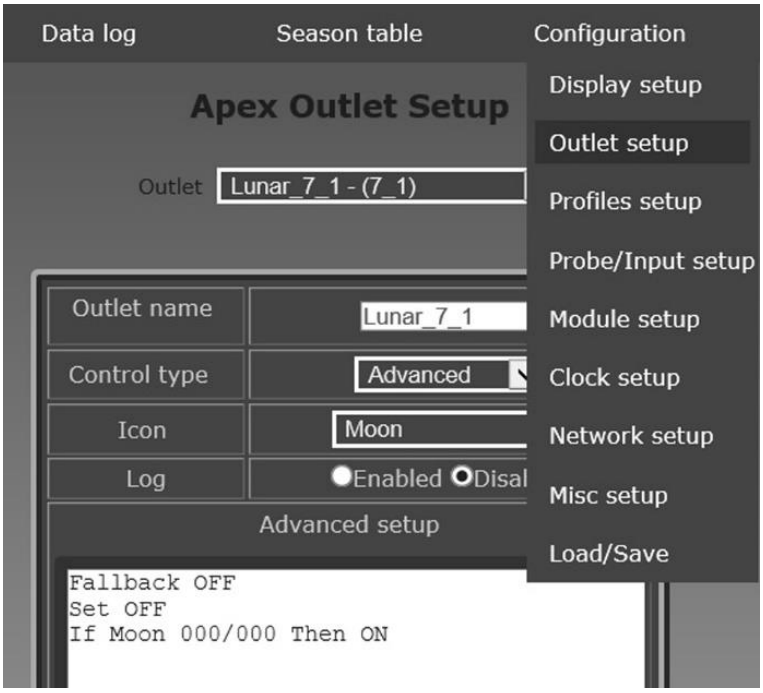


Appendix 8 Code for the Radion XR30w Pro LED outlet settings.

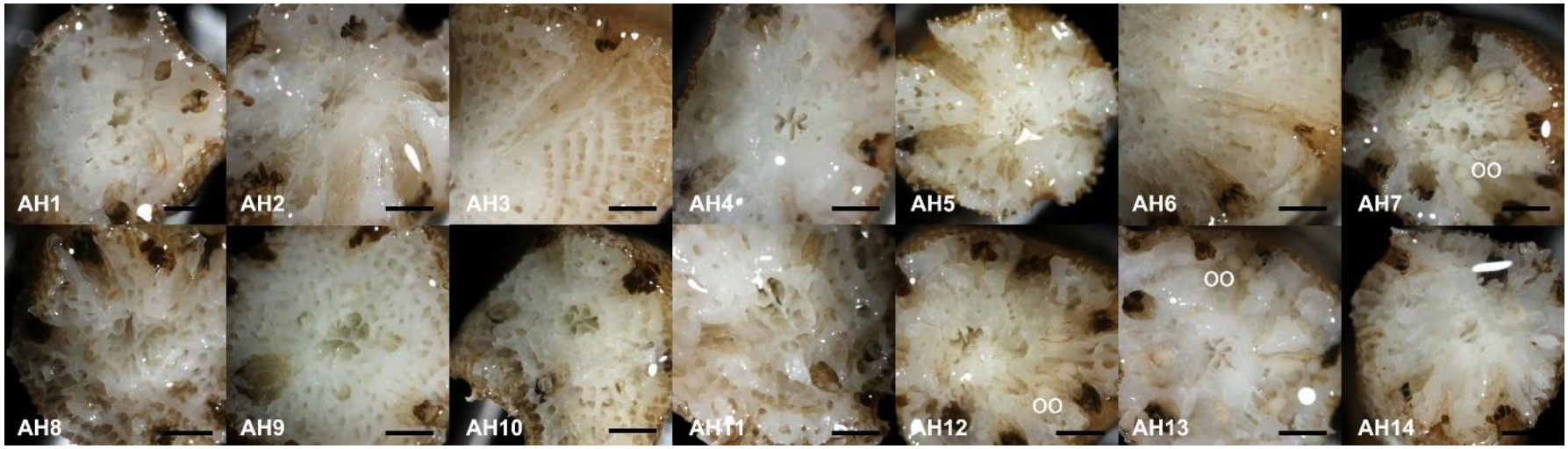
Programmed via Apex classic dashboard, drop down menu configuration, outlet setup to replicate annual seasonal photoperiod.

Appendix 9 Weekly Radion XR30w Pro % intensity changes populated from 22 year monthly average solar irradiance curves for Singapore and Australia (Figure 3.5).

Month	Week	Australian system profile light intensity % level	Singapore system profile light intensity % level
January	1	87	74
January	2	85	80
January	3	82	85
January	4	79	93
February	1	76	100
February	2	75	100
February	3	75	97
February	4	75	95
March	1	74	93
March	2	73	91
March	3	72	90
March	4	71	89
April	1	69	87
April	2	68	85
April	3	65	82
April	4	63	79
May	1	62	77
May	2	61	75
May	3	60	74
May	4	60	73
June	1	60	72
June	2	61	71
June	3	62	70
June	4	62	69
July	1	63	68
July	2	66	66
July	3	69	67
July	4	72	68
August	1	74	69
August	2	77	70
August	3	82	72
August	4	86	73
Sept	1	88	75
Sept	2	92	76
Sept	3	96	77
Sept	4	98	78
Oct	1	99	73
Oct	2	100	76
Oct	3	99	73
Oct	4	99	72
Nov	1	98	69
Nov	2	97	67
Nov	3	96	65
Nov	4	93	63
Dec	1	92	61
Dec	2	90	64
Dec	3	88	68
Dec	4	87	72

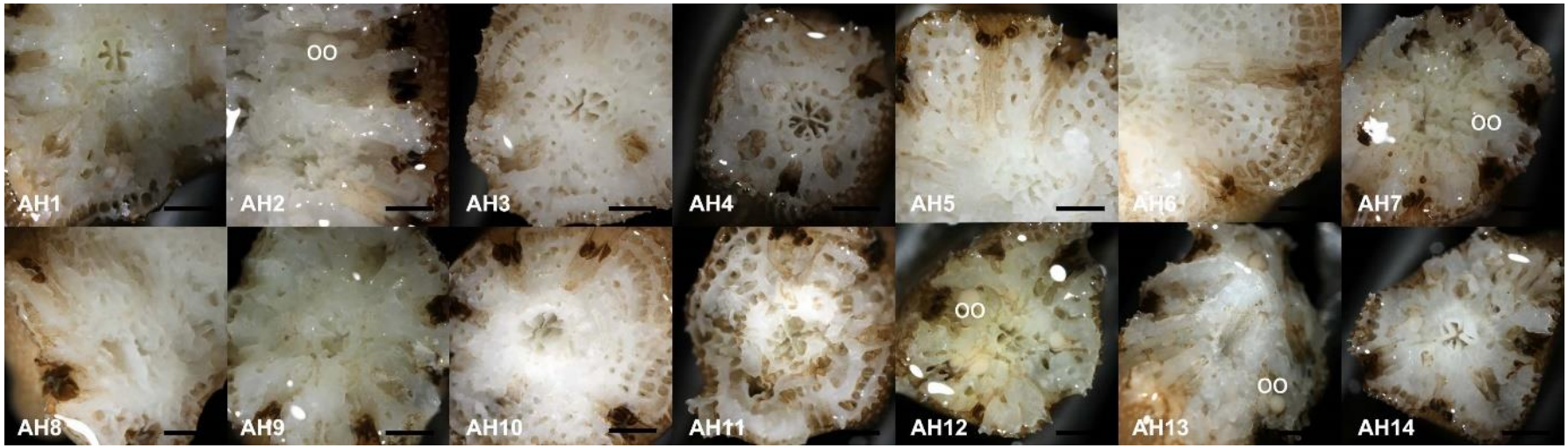


Appendix 10 Code for the Lunar simulator module programmed via Apex classic dashboard, to replicate moon phases.



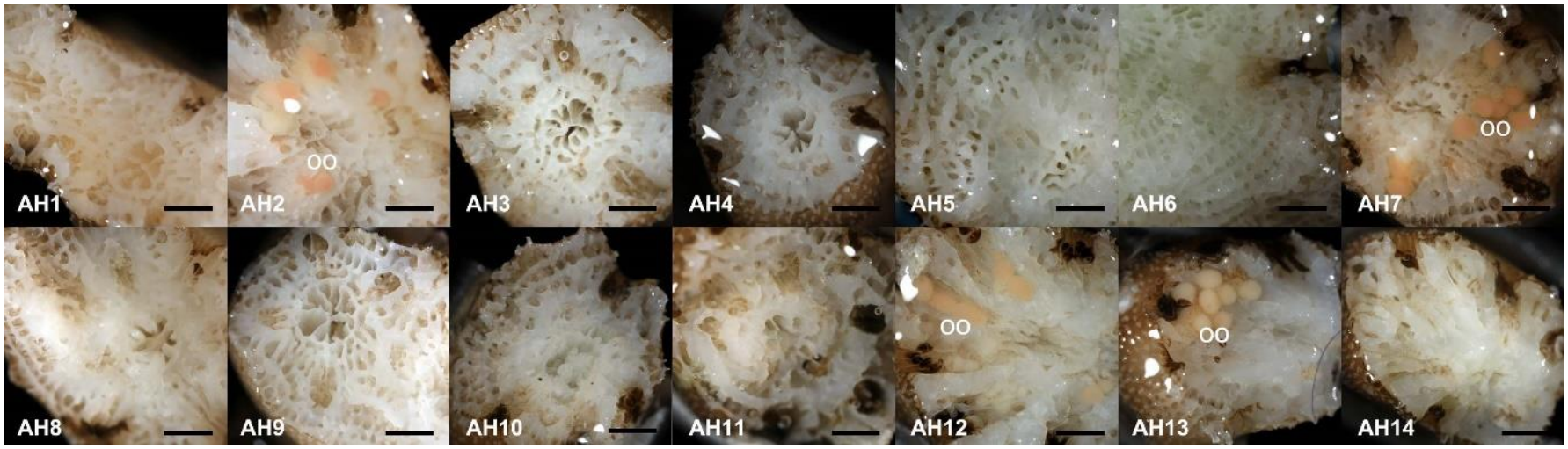
Appendix 11 Transverse sections of *Acropora hyacinthus* (colonies AH1-AH14) taken on 1st February 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



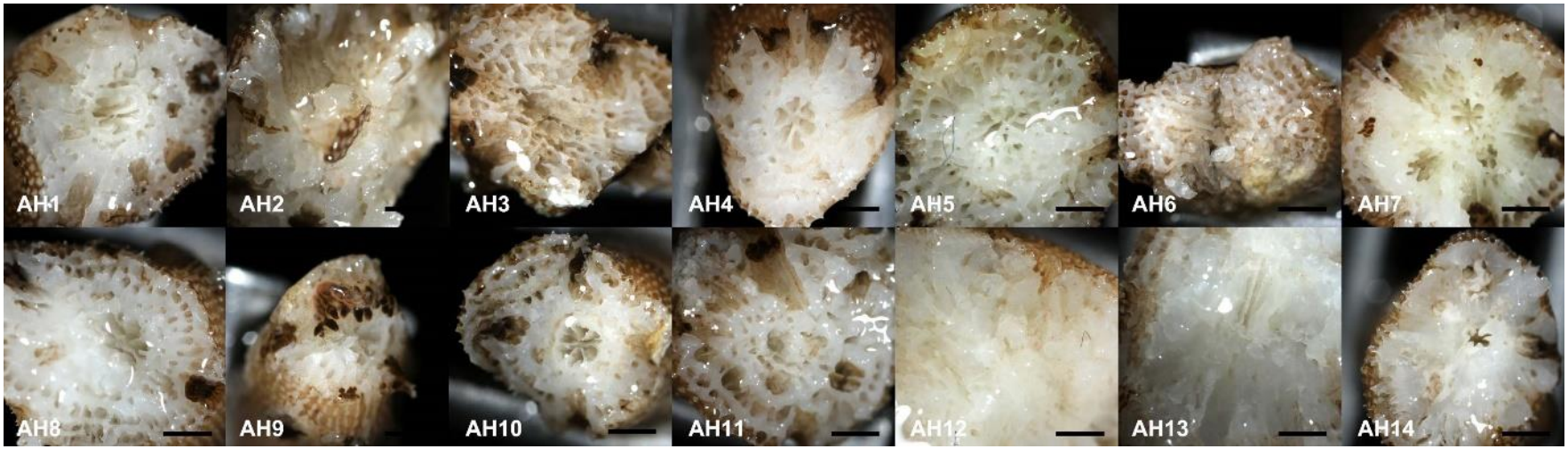
Appendix 12 Transverse sections of *Acropora hyacinthus* (colonies AH1-AH14) taken on 26th February 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



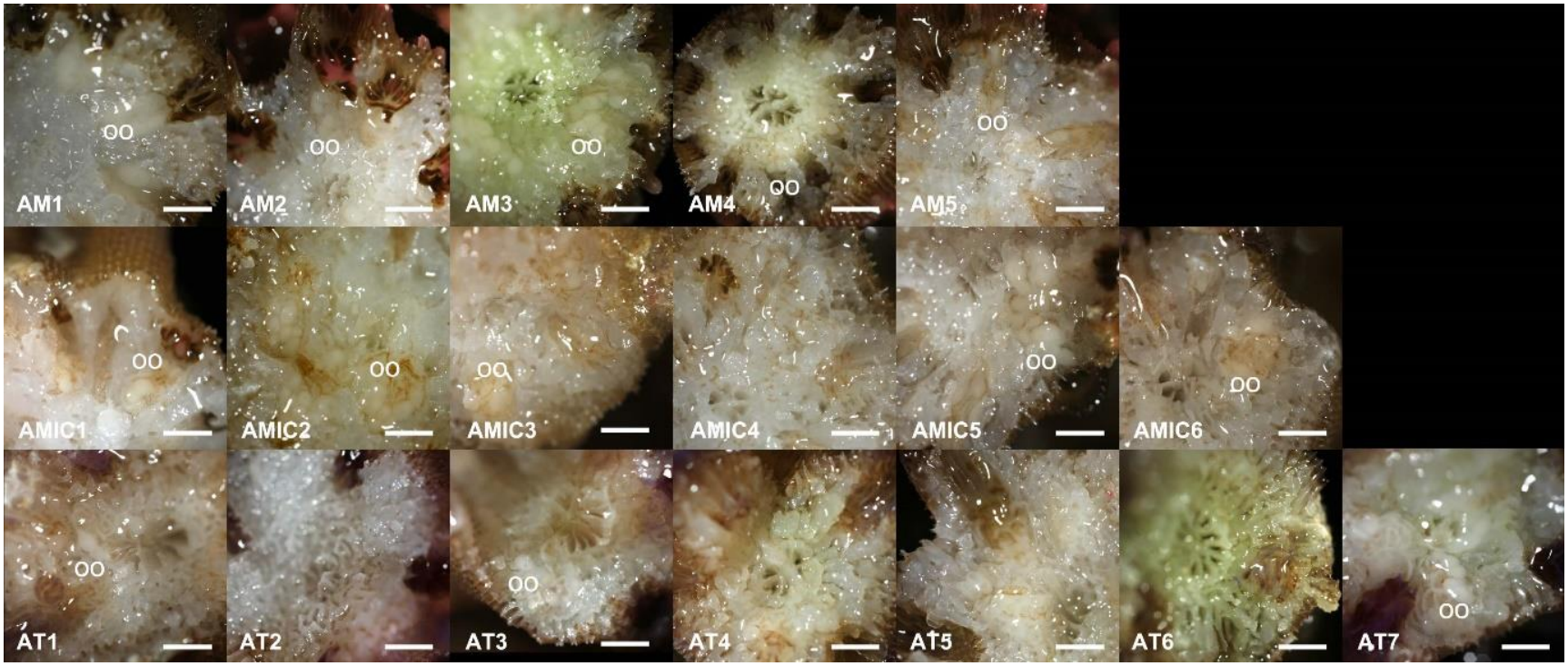
Appendix 13 Transverse sections of *Acropora hyacinthus* (colonies AH1-AH14) taken on 17th March 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



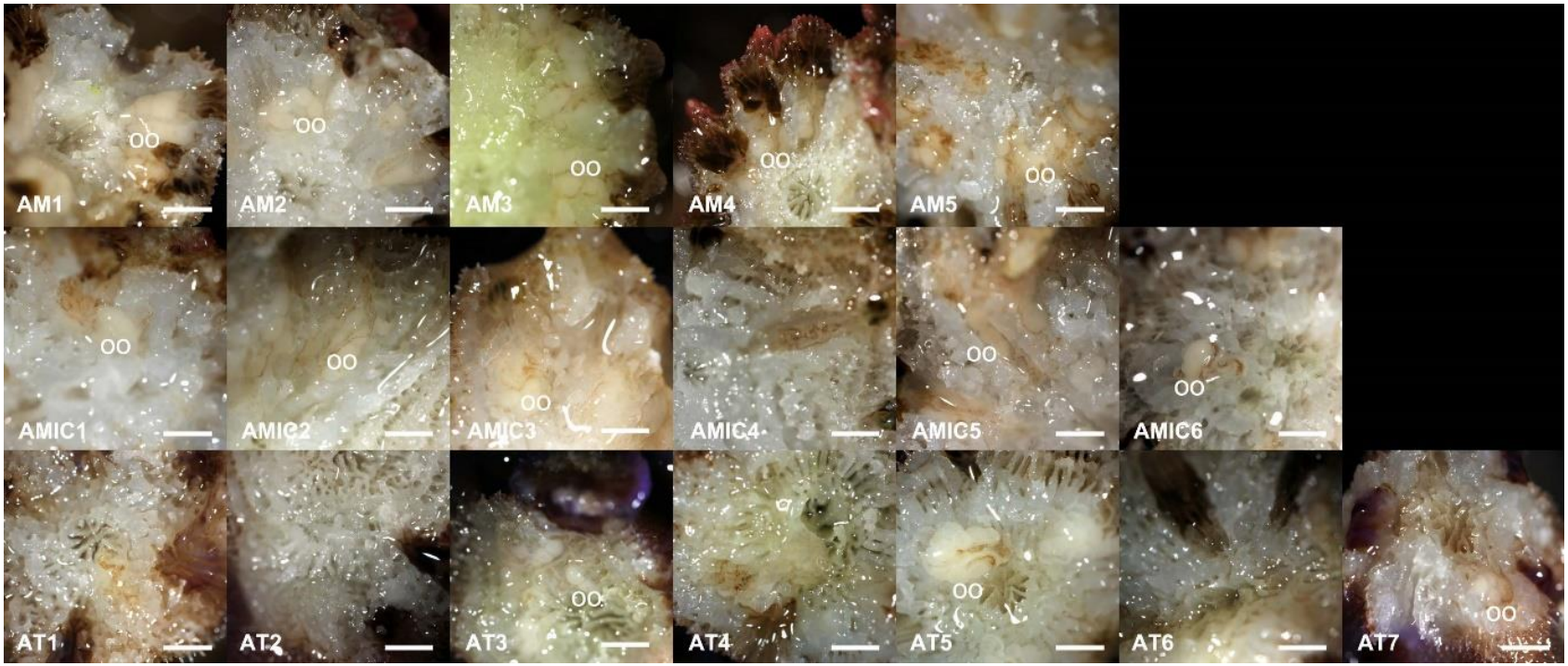
Appendix 14 Transverse sections of *Acropora hyacinthus* (colonies AH1-AH14) taken on 21st April 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



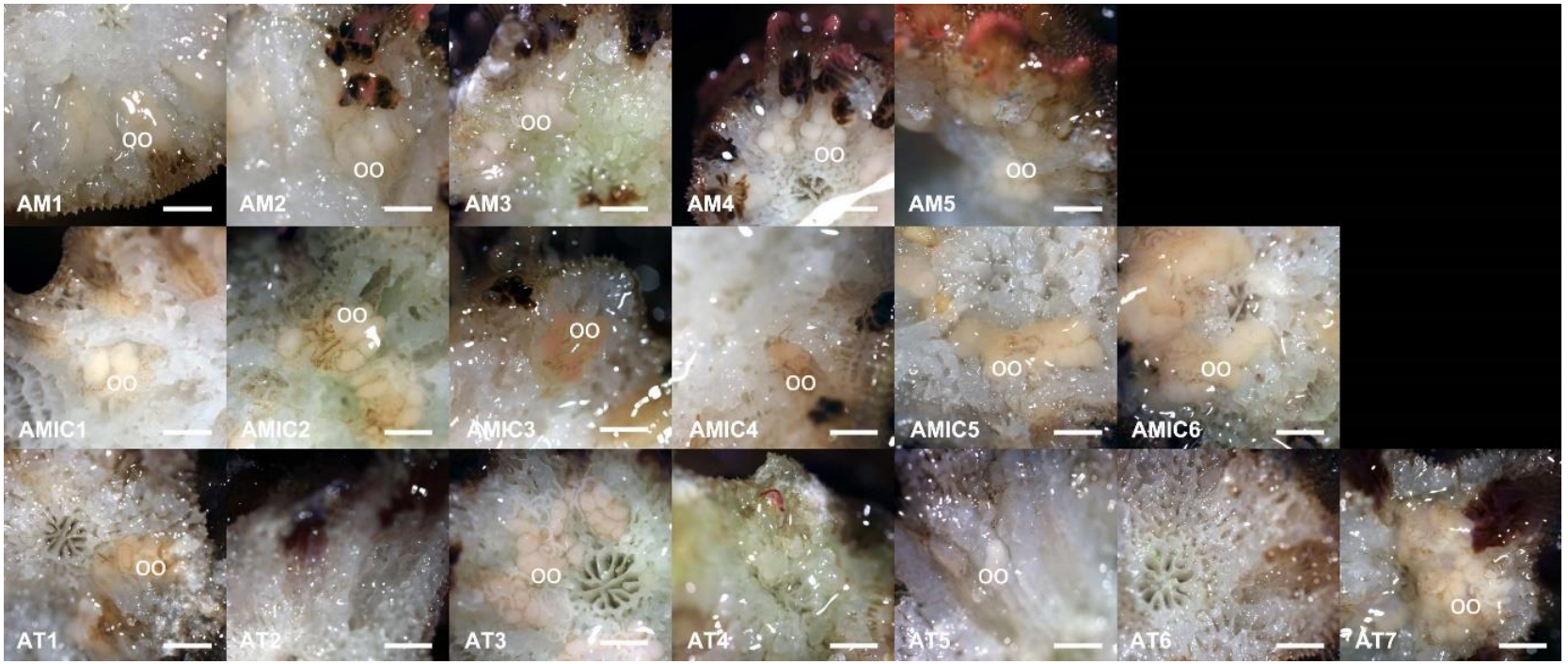
Appendix 15 Transverse sections of *Acropora millepora* (colonies AM1-AM5), *Acropora microclados* (colonies AMIC1-AMIC6) and *Acropora tenuis* (colonies AT1 – AT7) taken on 14th September 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



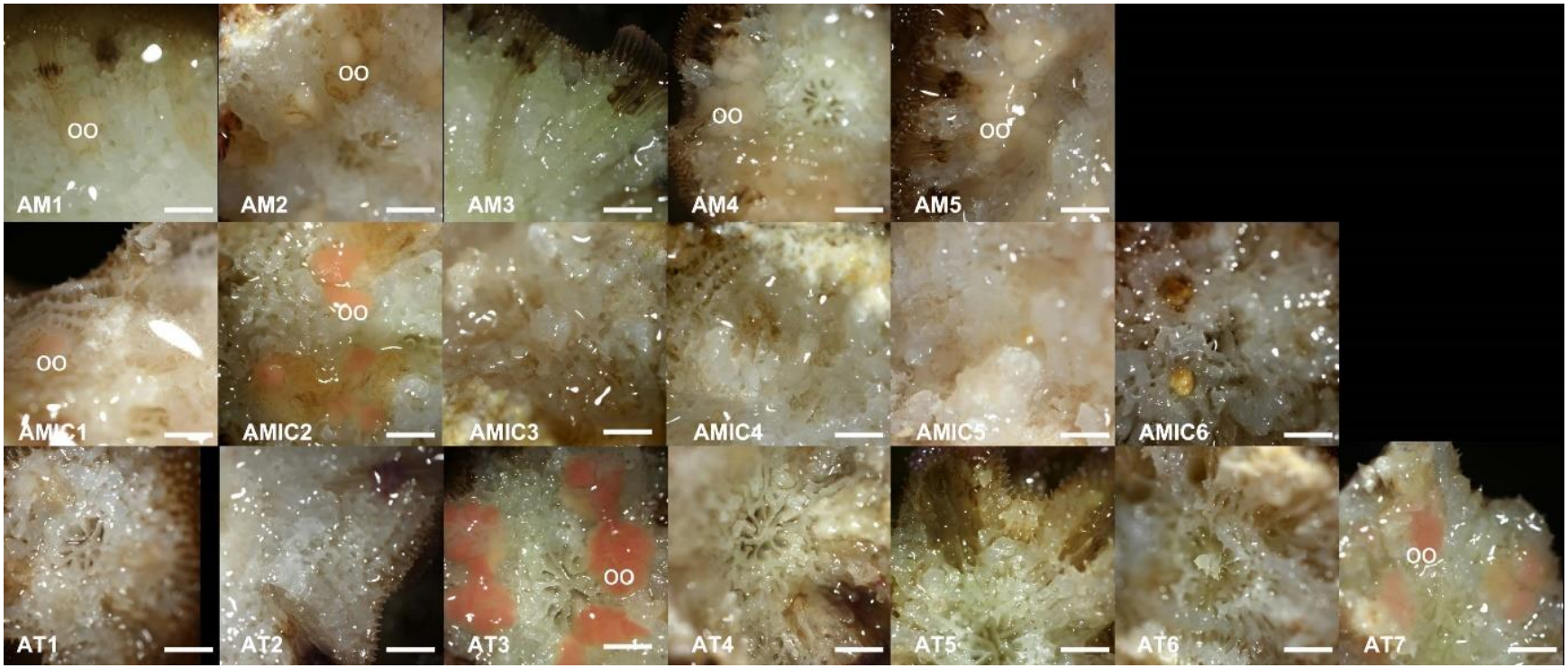
Appendix 16 Transverse sections of *Acropora millepora* (colonies AM1-AM5), *Acropora microclados* (colonies AMIC1-AMIC6) and *Acropora tenuis* (colonies AT1 – AT7) taken on 13th October 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



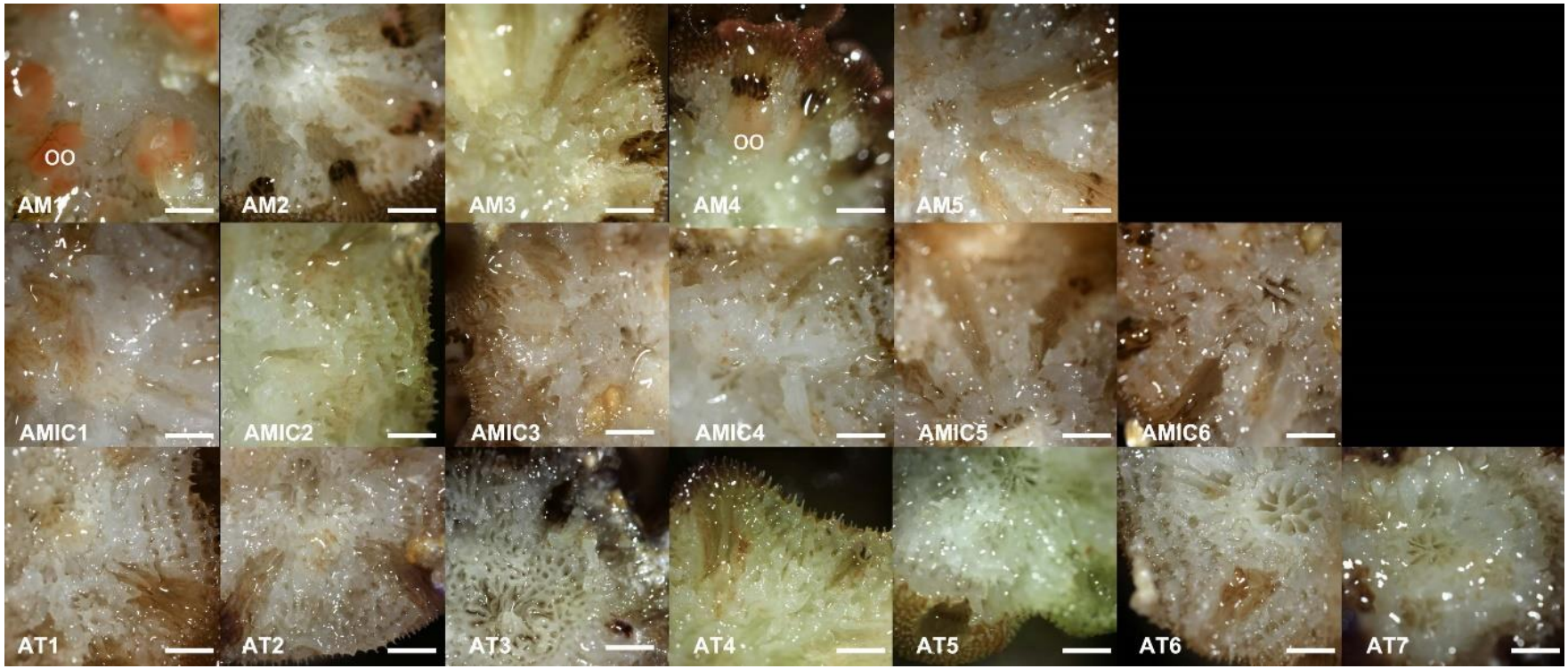
Appendix 17 Transverse sections of *Acropora millepora* (colonies AM1-AM5), *Acropora microclados* (colonies AMIC1-AMIC6) and *Acropora tenuis* (colonies AT1 – AT7) taken on 10th November 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



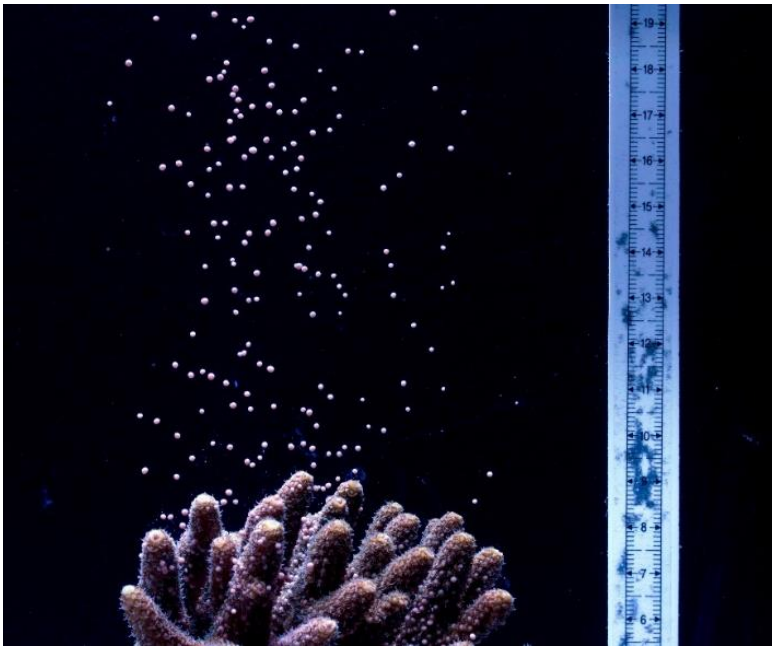
Appendix 18 Transverse sections of *Acropora millepora* (colonies AM1-AM5), *Acropora microclados* (colonies AMIC1-AMIC6) and *Acropora tenuis* (colonies AT1 – AT7) taken on 11th December 2016.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



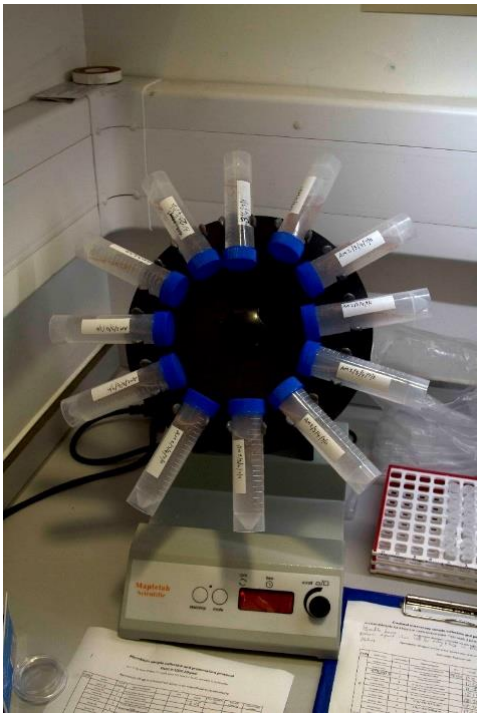
Appendix 19 Transverse sections of *Acropora millepora* (colonies AM1-AM5), *Acropora microclados* (colonies AMIC1-AMIC6) and *Acropora tenuis* (colonies AT1 – AT7) taken on 8th January 2017.

oo = oocytes. Scale 1mm. *All images taken by J Craggs*



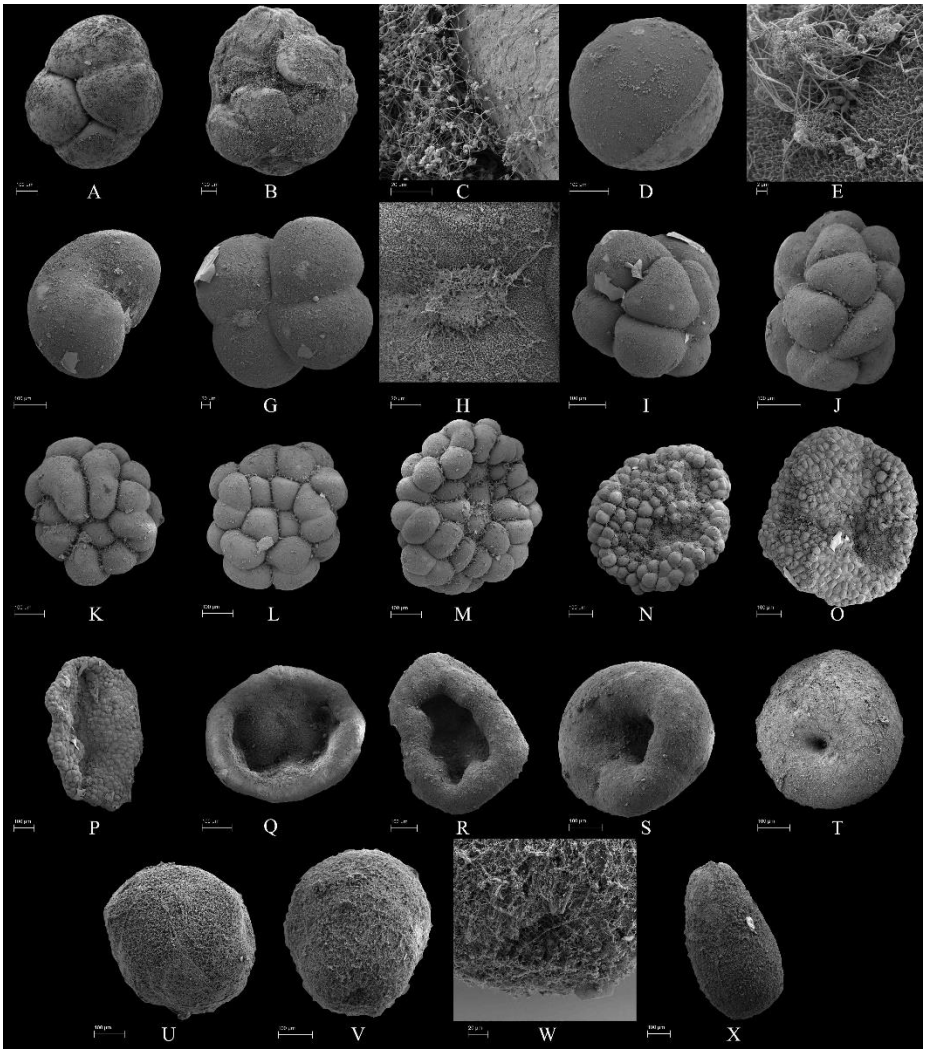
Appendix 20 *Acropora millepora* spawning in a photography aquarium. Bundle ascending rate were calculated from video footage shot during gamete release.

All images taken by J Craggs



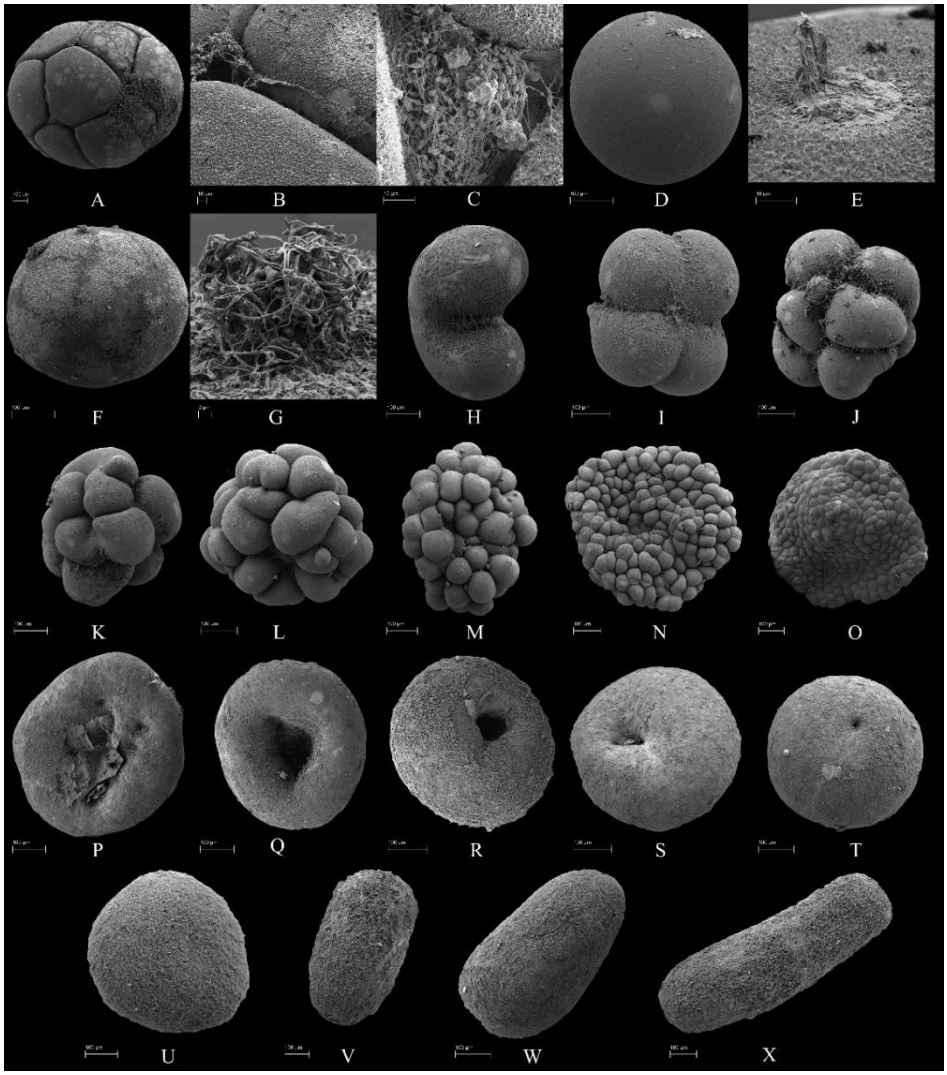
Appendix 21 Aliquots of newly released egg/sperm bundles are tumbled on a blood rotator to aid dissociation.

All images taken by J Craggs



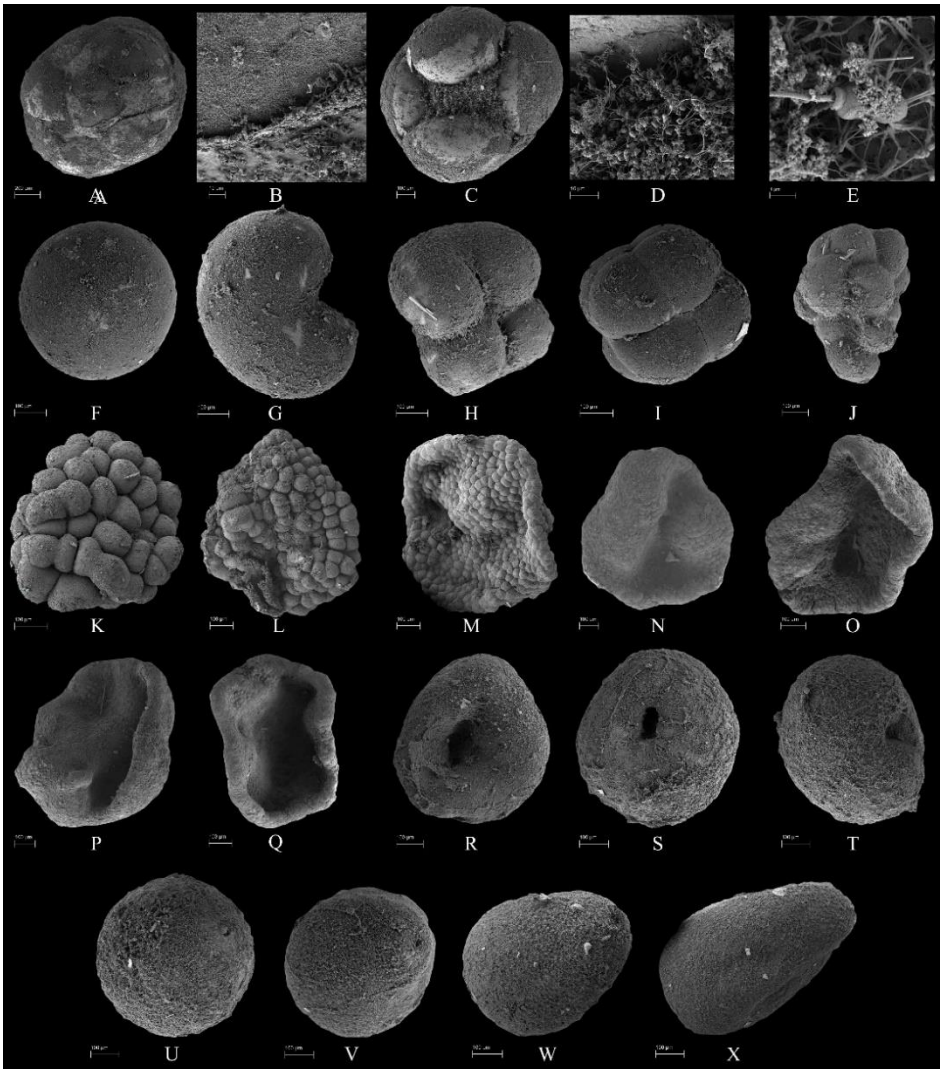
Appendix 22 SEM micrograph of *Acropora millepora* embryogenesis following completion of gametogenic cycle and spawning *ex situ*.

(A) Newly released egg sperm bundle; (B) Egg Sperm bundle dissociation; (C) Sperm swimming liberation during bundle dissociation as sulphated mucosubstances breaks down; (D) Newly separated oocytes; (E) Sperm penetrating oocytes ectoderm during fertilisation; (F) First cleavage / two-blastomere zygote (1 hour post fertilisation hpf); (G) Four-blastomere stage (2 hpf); (H) Sperm cells visible in the furrow of the first cell division. Close up of figure S4 G (2 hpf); (I) Eight-blastomere stage (2 hpf); (J) 16-blastomere stage (3 hpf); (K-N) Morula (4-6 hpf); (O-P) Prawn chip (7-8 hpf); (Q-S) Bowl stage (10-16 hpf); (T) Round stage. Gastrulation commences as the blastula rolls inward (28 hpf); (U-V) Tear drop stage (54-66 hpf); (W) Stomodeum formation via invagination of the ectoderm. Close up of Figure S4 V (66 hpf); (X) Planula elongation stage (102 hpf). Scale = 2µm (E), 20µm (C,G-H & W), 100µm (A-B,D,F,I-V & X). *All images taken by J Craggs*



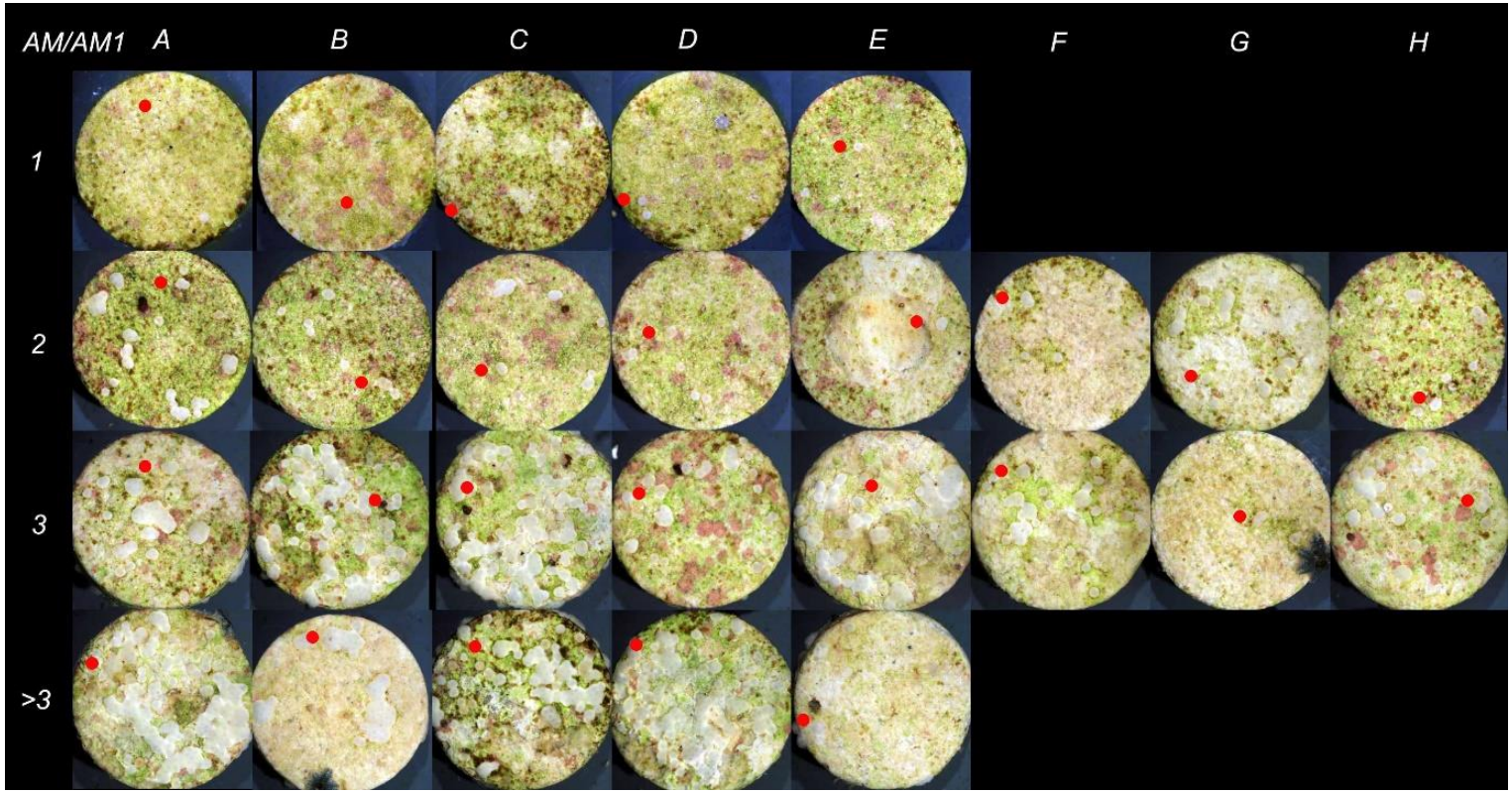
Appendix 23 SEM micrograph of *Acropora tenuis* embryogenesis following completion of gametogenic cycle and spawning *ex situ*.

(A) Newly released egg sperm bundle; (B) Sulphated mucosubstances holding egg/sperm bundle together; (C) Sperm swimming liberation during bundle dissociation as sulphated mucosubstances breaks down; (D) Newly separated oocytes. Polar body visible adjacent to the animal pole; (E) Polar body visible adjacent to the animal pole. Close up of figure S5 D; (F) Separate oocyte with undergoing fertilisation; (G) Sperm penetrating oocytes ectoderm. Close up of figure S5 F; (H) First cleavage / two-blastomere zygote (1 hour post fertilisation hpf); (I) Four-blastomere stage (2 hpf); (J) Eight-blastomere stage (3 hpf); (K) 16-blastomere stage (3 hpf); (L-N) Morula (4-6 hpf); (O-P) Prawn chip (7-8 hpf); (Q-R) Bowl stage (17 hpf); (S-T) Round stage. Gastrulation commences as the blastula rolls inward (22-29 hpf); (U) Tear drop stage (56 hpf); (V-X) Planula elongation stage (78-102 hpf). Scale = 2µm (G), 10µm (B-C&E), 100µm (A,D,F & H-X). *All images taken by J Craggs*



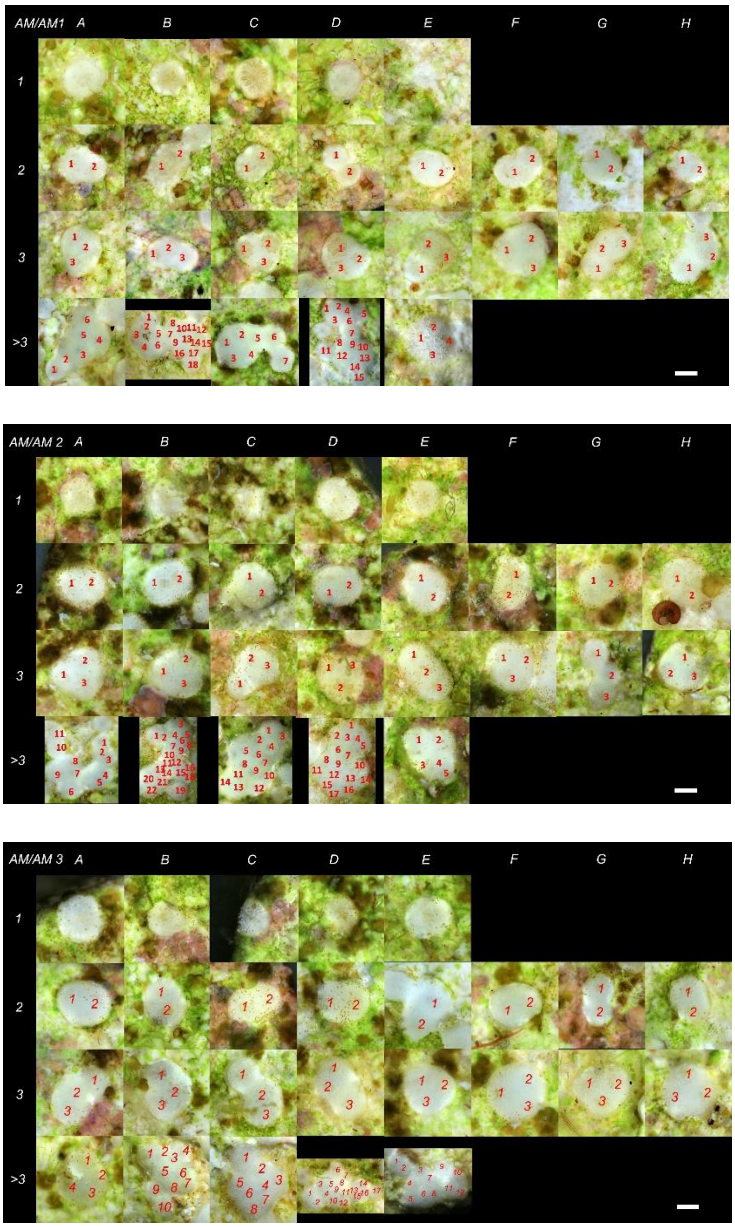
Appendix 24 SEM micrograph of *Acropora anthocercis* embryogenesis following spawning *ex situ*.

(A) Newly released egg sperm bundle; (B) Sulphated mucosubstances holding egg / sperm bundle together; (C) Egg Sperm bundle dissociation; (D) Sperm swimming liberation during bundle dissociation as sulphated mucosubstances breaks down; (E) Sperm penetrating oocytes ectoderm during fertilisation; (F) Separate oocyte with undergoing fertilisation; (G) First cleavage / two-blastomere zygote (2 hrs post fertilisation (hpf)); (H) Four-blastomere stage (3 hpf); (I) Eight-blastomere stage (4 hpf) ; (J) 16-blastomere stage (6 hpf); (K-L), Morula (6-8 hpf); (M-Q) Prawn chip (8-11 hpf); (R) Bowl stage (16 hpf); (S-U) Round stage. Gastrulation commences as the blastula rolls inward (20-28 hpf); (V-W) Tear drop stage (42-66 hpf); (W-X) Planula elongation stage (78 hpf). Scale = 1µm (E), 10µm (B&D), 100µm (F-X) 200µm (A). *All images taken by J Craggs*



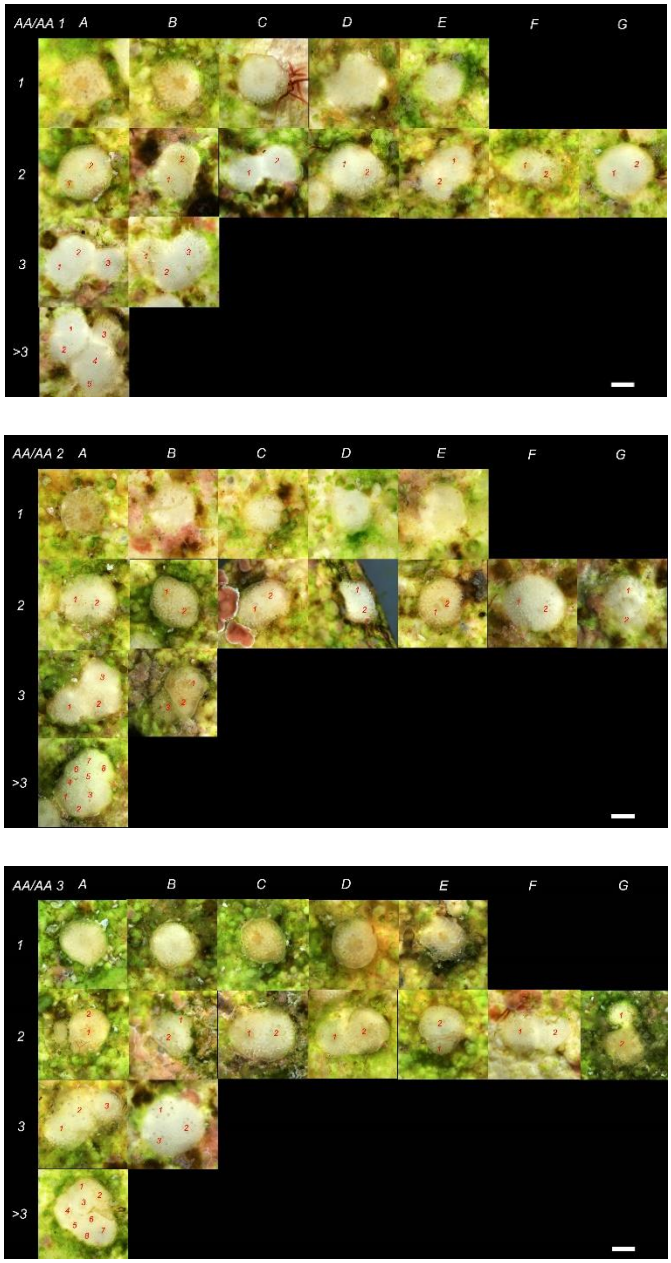
Appendix 25 Entity class map to follow specific entities over the study period. Red dot situated to the left of the entity.

Genotype's numbers within the entity class show on the y axis (1 genotype, 2 genotype, 3 genotype and >3 genotype entities), entity class replication on the x axis (A-H). *Acropora millepora* / *A. millepora* replicate 1 represented. *All images taken by J Craggs*



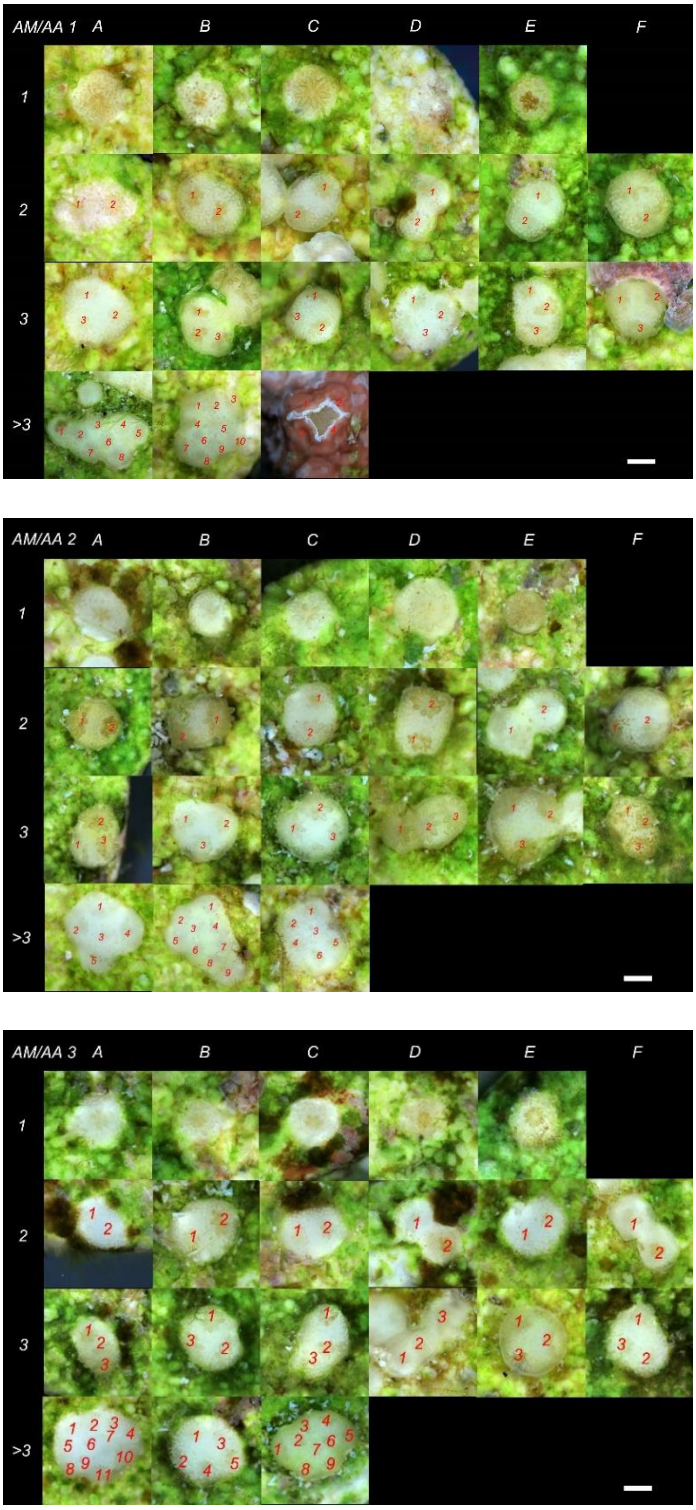
Appendix 26 *Acropora millepora* / *A. millepora* (AM/AM) genotype maps of entity classes.

Genotypes numbers within the entity class show on the y axis (1 genotype, 2 genotype, 3 genotype and >3 genotype entities) and express numerically, entity class replication on the x axis (A-H). *All images taken by J Craggs*



Appendix 27 *Acropora anthocercis* / *A. anthocercis* (AA/AA) genotype maps of entity classes.

Genotype's numbers within the entity class show on the y axis (1 genotype, 2 genotype, 3 genotype and >3 genotype entities) and express numerically, entity class replication on the x axis (A-G). *All images taken by J Craggs*



Appendix 28 *Acropora millepora* / *Acropora anthocercis* (AM/AA) genotype maps of entity classes.

Genotype's numbers within the entity class show on the y axis (1 genotype, 2 genotype, 3 genotype and >3 genotype entities) and express numerically, entity class replication on the x axis (A-F). *All images taken by J Craggs*

Appendix 29 Coral size frequency distributions at 180 days in four sea urchin grazing treatments.

(non-grazing control, low grazing density (4 urchins = 16.67/m^2), medium grazing density (nine urchins = 37.50/m^2), high grazing density (18 urchin = 75/m^2).)

Coral diameter (mm)	non-grazing	low	medium	high
[0,10]	60	129	95	156
[10,20]	5	82	91	122
[20,30]	0	45	49	83
[30,40]	0	18	28	38
[40,50]	0	14	22	35
[50,60]	0	4	8	9
[60,70]	0	2	7	7
[70,80]	0	0	6	6
[80,90]	0	0	1	4
[90,100]	0	0	0	2
[100,110]	0	1	0	1
[110,120]	0	0	0	0
[120,130]	0	0	1	0