Improving coral aquaculture for reef conservation and the aquarium trade

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Improving coral aquaculture for reef conservation and the aquarium trade

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I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.
I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

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Abstract

Corals are under threat worldwide. Many regions including the Great Barrier Reef have lost large areas of coral cover from a variety of causes, such as ocean warming, overfishing, eutrophication and sedimentation. Coral aquaculture may play a role in reef conservation by contributing large quantities of live material for restoration projects. Improving coral aquaculture will also have a positive influence on the aquarium trade, drug discovery and experimental research. Despite this potential, less than 25% of coral species have been cultured, and many aspects of the aquaculture process need to be improved by determining the combinations of factors that optimise coral growth. The overarching aim of this thesis was to use a robust experimental approach to develop protocols that optimise the culturing of three different coral species (Acropora millepora, Hydnophora rigida and Duncanopsammia axifuga). To achieve this, I examined different ways to grow corals, testing hypotheses about feeding, alternate ways to fix coral fragments to the substrate, as well as ways to enhance resilience against temperature stressors using a lipid-enriched diet. Key findings of the study were that: (1) there are no significant differences between day and night feeding for the three studied coral species (p > 0.06); (2) the optimum concentration of food to achieve maximum feeding rates was determined for each of the three corals species (> 50 ind ml⁻¹); (3) fragments without polyps can be used in aquaculture as in a short time period they develop new polyps (~20 to 40 days); (4) fragments fixed upside-down attach faster and wider over the substrate (87%); and (5) a lipid enriched-diet enhances resilience against temperature stressors. Finally, by improving culture methods for several key species of coral, the work may contribute to successful coral reef restorations and help develop sustainable coral aquaculture for the aquarium trade.
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Statement of contribution

Chapter 2

T.A. participated during all stages of the development of this paper and provided an overall contribution greater than that of any co-author. T.A. and R.S. performed the experiments and laboratory work. T.A. ran the statistical analyses, and wrote the first draft of the manuscript which was revised with feedback from the co-authors.

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Abbreviations

A: Acropora
Art: Artemia salina
BW: buoyant weight
CG: coral growth
C.I: confidence intervals
Chl: chlorophyll
D: Duncanopsammia
d.f.: degrees of freedom
FA: fatty acid
FAME: fatty acid methyl esters
FSW: filtered sea water
GC: gas chromatography
GC-MS: gas chromatography–mass spectrometry
H: Hydnophora
HPLC–HRMS: high performance liquid chromatography–high resolution mass spectrometry
HRMS: High resolution mass spectrometry
Km: half-saturation constant
MUFA: monounsaturated fatty acid
PL: phospholipids
PUFA: polyunsaturated fatty acid
Ret: retention
rpm: revolutions per minute
SD: standard deviation
SE: standard error
SFA: saturated fatty acid
Vmax: maximum feeding rate
Chapter 1 - Introduction
Coral reefs are the largest living structures in the world, providing valuable services for the health of ocean ecosystems, as well as many goods and services to human populations (Veron 2000). This chapter introduces the current key environmental issues related to coral reefs and problems associated with coral trade. The biology of corals is discussed in the context of relevance for aquaculture and reef restoration (e.g. reproduction and nutrition), as well as different techniques necessary to further improve coral aquaculture (e.g. growth measurements).

1.1 The importance of coral reefs and threats to their persistence

The scleractinians, or stony corals, are represented by over 300 species (Veron 2000). Under the right conditions, stony corals can combine to build reefs (Birkeland 1996), which act as a physical barrier for currents and waves, preparing the environment for the proliferation of mangroves and seagrass (Moberg and Folke 1999). Coral reefs are one of the most diverse ecosystems on earth (Birkeland 1996). Approximately 109 countries are located adjacent to coral reefs and around 450 million people rely on these ecosystems for food and employment (Carpenter et al. 2008; Pandolfi et al. 2011); it has been calculated that around 10% of the world population is currently benefiting directly from coral reefs (Osinga et al. 2011).

Coral reefs are threatened by nutrient loading, sedimentation and over fishing, as well as ocean warming, acidification and sea level rise (Anlauf et al. 2011; Hoegh-Guldberg et al. 2007; Hughes et al. 2017). The stressors interact (e.g. synergistic or antagonistic) to impact reefs around the world and vary among regions (Fabricius 2005; O’Neil and Capone 2008; Pandolfi et al. 2011). In all likelihood, rather than becoming extinct, corals will change and adapt to new environmental conditions, with some species doing better than others (Hughes et al. 2003). Nonetheless, it is presently unknown how many species would be capable of adapting to the predicted ocean warming scenarios (Palumbi et al. 2014). It is also unknown whether corals can gradually adapt to future changes in the carbonate chemistry of the oceans (Kleypas et al. 1999).

Another important consideration for coral reef management is the multi-million dollar aquarium industry, which harvests from the wild around 98% of the corals sold (Moe 2001). In some areas, corals are also used for construction (Brown and Dunne 1988; Caras and Pasternak 2009). For example, as much as 25,000 tons were mined in a single year in the Maldives for this industry (Delbeek 2001), and it will take at least 50 years for these mined reefs to recover their original conditions (Brown and Dunne 1988). Furthermore, the capacity of coral reefs to recover naturally from aquarium collection is
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decreasing due to increasing environmental stress, creating the need for an effective coral aquaculture industry (Pomeroya *et al.* 2006).

1.2 An overview of coral biology

1.2.1 Taxonomy, diversity and physiology

Scleractinia, or stony corals belong to the Phylum Cnidaria, which includes 25 orders, showing a similar general body plan among corals, soft corals, hydroids, jellyfish, and sea anemones. The Scleractinian (or hard skeleton corals) order has 17 families, 107 genera and over 300 species (Veron 2000).

The animal polyp is the anatomical unit of corals, consisting of a main body or coelenteron with cylindrical structure and a central mouth surrounded by tentacles (Veron 2000). The coelenteron is a complex structure, divided by vertical mesenteries radially arranged, which provide an extended surface area for digestion, photosynthesis and respiration. Almost all reef-building corals are colonial organisms, comprising polyps linked together by the coenosarc, through which water and nutrients are transported (Veron 2000, Sorokin 1995). Both the coenosarc and polyp tissues consist of two epithelial layers called the epidermis or ectoderm, and gastrodermis or endoderm (Veron 2000, Sorokin 1995). These two epithelial layers are separated by the mesoglea, a connective extracellular matrix highly hydrated and composed of collagen fibres (Tambutté *et al.* 2011; Veron 2000).

Despite being very simple organisms, corals have well developed nervous and muscular systems. A nerve, composed of ectodermal and gastrodermal cells, has connections to a variety of cells responsible for detecting mechanical, chemical and light stimuli (Veron 2000). Meanwhile, the muscular system allows polyps to move in response to signals from the nervous system, which are transmitted from polyp to polyp (Veron 2000). The skeletal morphology of corals is genetically controlled, yet some species show substantial variations in response to environmental parameters, known as phenotypic plasticity (Garland and Kelly 2006).

Scleractinian corals have a strong metabolic dependence on a long-term evolutionary endosymbiosis to fix CO$_2$ and to deposit aragonite skeletons (Shinzato *et al.* 2011). Corals are the product of a symbiotic relationship between single celled photosynthetic algae (*Symbiodinium*) and animals. These *Symbiodinium* obtain a stable and sunlight-exposed medium in which to live, while the coral animal receives an enhanced capacity to remove metabolic waste, to calcify, to concentrate and recycle limited nutrients such as nitrogen and phosphate, as well as a direct source of more
complex nutrients (Veron 2000). So, this symbiosis allows corals to exist in very low-nutrient environments (Grover et al. 2008; Veron 2000), and to augment the rate of skeletal calcification, which can be two to three times faster in the light than in the dark (Muscatine et al. 2005).

The variety of species within the *Symbiodinium* genus reflects functional diversity towards factors such as resource exchange between symbiotic partners, thermal tolerance, symbiont transmission strategies and mode of symbiont reproduction, or even the capability of the symbiont to persist outside the host in a free-living state. Corals often harbour more than one clade of *Symbiodinium*, which theoretically creates potential physiological plasticity, by shuffling symbiont types or switching from one dominant type to another that is more physiologically suited to particular environmental conditions (Lesser et al. 2013).

Fluorescent pigments provide the brilliant colours in corals and also help to control the light environment within the coral tissue, increasing light accessibility in low light conditions, but also protecting against excess UV and assisting to prevent bleaching during stress periods (Salih et al. 2000). *Symbiodinium* provide the main pigments for corals (principally chlorophyll *a*, and secondary carotenoids, peridinin, chlorophyll *C*₂, and xanthophyll) (Jeffrey and Haxo 1968). Environmental conditions directly affect the amount of pigments and their colour intensity; for this reason coral pigmentation has been used recently as a water quality bioindicator (Cooper and Fabricius 2012). However, recent evidence shows that, at least for mesophotic deep reefs (40-100m), light availability does not limit fluorescent pigment concentration (Eyal et al. 2015), and some experiments have indicated that nutrient concentrations directly affect the densities of the *Symbiodinium*, even developing internal “algal blooms” at high ammonia concentration, resulting in corals with more pigmentation (Hoegh-Guldberg and Smith 1989).

**1.2.2 Distribution.**

Species level diversity is driven by oceanic circulation, niche differentiation, and geographic and evolutionary changes over time (Veron 2000); yet, the distribution of shallow-water corals is predominantly regulated by water temperature, and secondarily by light availability, aragonite saturation, salinity and low nutrient concentration (Couce et al. 2012). However, other factors like pH, water movement, sedimentation, dissolved oxygen and UV radiation also influence coral distribution (Osinga et al. 2011). Additionally, the geological development of a region determines the number and kinds of species occurring across it, and as a consequence, regions with different histories can
support different numbers of species, which is usually reflected in the richness of the local community (Cornell and Karlson 2000).

Coral reefs are found over a wide range of environments (among certain limits), with tropical coral reefs usually restricted to warm water and bright light (e.g. Great Barrier Reef and Caribbean) and temperate reefs where the water can reach 16ºC in winter (e.g. Persian Gulf, Gulf of Mexico) (Delbeeck and Sprung 1994). In Australia, the coral communities of Lord Howe Island are considered one of the southernmost coral reefs in the world (Veron 1986).

1.2.3 Coral reproduction

Information available about coral reproductive biology is considered limited, and few species have been studied well (Weil and Vargas 2010). To illustrate how incomplete our understanding of coral reproduction is, it was widely accepted that all gametes are released from the oral opening, but eggs of *Stephanocoenia intercepta* (Lamarck 1836) have recently been observed being released from the tentacle tips (Vermeij *et al.* 2010). Coral species show several reproductive strategies, alternating between sexual and asexual modes, depending on environmental conditions. Figure 1.1 illustrates relevant reproductive processes for coral aquaculture and restoration projects.

**Figure 1.1.** Coral reproduction cycle: sexual and asexual reproduction.

1.2.3.1 Sexual reproduction

The principal advantage of this reproductive mode for aquaculture or reef restoration projects is that it improves genetic variability (Harrison and Wallace 1990). However, environmental conditions can affect the development and duration of gametogenesis between species (Szmant 1986) and influence the development of reproductive organs (Veron 2000).
Once gametes are mature, the release of eggs and sperm occurs amongst the broadcasting species in the spawning season; however, brooding species fertilize eggs inside their polyps, and the larvae are somewhat developed before the release of planulae or embryos into the water (Harrison and Wallace 1990). Some larvae can potentially travel long distances before settlement (Gleason and Hofmann 2011). For example, the larvae of *Pocillopora damicornis* (Linnaeus 1758) can still be found in the plankton after 100 days (Richmond 1987).

Reproductive strategies vary among scleractinian species, which sometimes show no fixed reproduction mode within the same species. A clear example is observed in *P. damicornis*, considered a simultaneous hermaphrodite and an internal brooder in many areas, but a brooder and broadcast spawner in Western Australia (Ward 1992). Similarly, *Pavona gigantea* Verrill, 1869, is gonochoric and co-sexually hermaphroditic (Rodríguez-Troncoso *et al.* 2011).

Among the disadvantages of this reproductive mode for aquaculture and reef restoration is that reef-building species are showing an increased and unparalleled deterioration in recent decades, with growing uncertainties surrounding whether the amount of sexual recruitment is enough to sustain coral reefs (Gleason and Hofmann 2011). This is largely because it is a slow process and corals can take several years to be mature and contribute new recruits to the reefs (Guest *et al.* 2014). Additionally, coral larvae survival and settlement rates can be low (Rodríguez *et al.* 2009) and sexual maturity is normally reached after three years (Guest *et al.* 2014). However, recent studies have shown that it may be possible to re-establish a breeding population of *Acropora tenuis* on degraded reefs using enhanced larval supply (dela Cruz and Harrison, 2017), but these methods are in the early stages of development and have not yet been applied to other reef-building corals.

### 1.2.3.2 Asexual reproduction

This reproductive mode, also known as “clonal propagation”, can occur by detachment of a tissue fragment, release of an asexual planula, budding, fission, polyp bail-out and cyst formation (Foster *et al.* 2007; Gleason and Hofmann 2011). Fragmentation is the most common way that asexual reproduction occurs in nature, and the most successful coral species are all adapted to fragmentation (Highsmith 1982), as it represents a suitable strategy to deal with many threats, such as predation, storms, hurricanes and bio-erosion (Smith and Hughes 1999). This phenomenon has been well studied in plants that are not able to complete a normal reproductive cycle, and as a consequence, switch to an asexual reproductive mode as an adaptation that allows the
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population to persist even during prolonged times of negative population growth. Although, it is believed that all corals can produce asexual budding of individual coral polyps (Jackson 1977), monoclonal dominance of the population has been observed and sexual extinction is a significant concern for conservation (Honnay and Bossuyt 2005) and might be considered as a disadvantage of asexual reproduction in restoration projects.

Nevertheless, fragmentation is a common tool used to obtain several replicates of the same genotype in coral experiments (Keshavmurthy et al. 2013; Rocha et al. 2013b; Towle et al. 2015), and also to maximize the production of nubbins in restoration projects (Forrester et al. 2014; Gomez et al. 2014; Mercado-Molina et al. 2015). For example, fragmentation in ex-situ cultures has demonstrated that three colonies can produce as many as 800 nubbins. Nubbins are defined as small fragments, down to the size of a single polyp (Shafir et al. 2001).

1.3 Coral aquaculture overview

Coral aquaculture is a rapidly emerging industry (Osinga et al. 2011); although most coral research is focused on ecology (Anthony 1999; Fabricius 2005; Rodriguez et al. 2009; Rogers 2004), physiology (Gori et al. 2014; Iluz and Dubinsky 2015; Sheridan et al. 2014), genetics (Boulotte et al. 2016; van Oppen et al. 2015), conservation (Cabaitan et al. 2015; Cruz and Harrison 2017; Rinkevich 2014) and climate change impacts (Grottoli et al. 2014; Hughes et al. 2017; Palumbi et al. 2014; Towle et al. 2015). For this reason, coral aquaculture is still in its infancy (in production and research) when compared to other sectors, such as fish or shrimp aquaculture (Leal et al. 2016a; Olivotto et al. 2011).

The first experiments with coral propagation are believed to have been undertaken in 1956 at the New Caledonia Aquarium (Delbeek 2001). Since then, there have been many improvements to coral aquaculture techniques, and it is now possible to reproduce, grow and maintain corals in aquaria (Barton et al. 2017; Borneman and Lowrie 2001; Delbeek 2001; Rocha et al. 2015). Furthermore, several species have been effectively cultured in nurseries, and then transplanted back to the wild using a variety of approaches (Rinkevich 2014). Coral aquaculture can be divided into two principal categories, in situ and ex situ, described below.

1.3.1 Coral aquaculture in situ

Mariculture involves the culture of a marine organism in its natural environment, and it is characterized by having all the benefits provided by the ecosystem (e.g. food,
sunlight, water quality and aeration), but also the disadvantages such as storms, pollution, competition, predation and disease (Barton et al. 2017; Leal et al. 2016a; Sheridan et al. 2013). Although in situ cultures are generally low cost operations, the reliance on natural conditions means that rates of coral growth cannot be enhanced (Borneman and Lowrie 2001). This type of coral aquaculture uses fragmentation as a preferred technique to reproduce corals that are later sold to the aquarium trade, or used to restore damaged reefs (Barton et al. 2017; Delbeek 2001; O’Donnell et al. 2017).

Large amount of coral aquaculture research is linked to coral restoration attempts (Barton et al. 2017; Boch and Morse 2012; Horoszowski-Fridman et al. 2015; O’Donnell et al. 2017). Diverse in situ culturing techniques have been investigated to provide corals for restoration (Barton et al. 2017; dela Cruz et al. 2015; Nedimyer et al. 2014). Reef rebuilding efforts are popular around the world, with published accounts in Singapore (Bongiorni et al. 2011), the Philippines (Gomez et al. 2014; Levy et al. 2010; Shaish et al. 2008; Shaish et al. 2010), Israel (Amar and Rinkevich 2007; Horoszowski-Fridman et al. 2011; Linden and Rinkevich 2011), Hawaii (Piniak and Brown 2008), the Pacific coast of Costa Rica (Guzmán 1991), Puerto Rico (Mercado-Molina et al. 2015), the British Virgin Islands (Forrester et al. 2014; Forrester et al. 2011), Florida (Williams and Miller 2010), the US Virgin Islands (Garrison and Ward 2008), Japan (Omori 2010), Tanzania (Mbije et al. 2013) and Mexico (Linan-Cabello et al. 2011).

Coral attachment is considered a critical step in coral reef restoration projects and it is well known that after corals are fully attached their mortality rate decreases significantly (Guzmán 1991; Forrester et al. 2011). In the restoration attempts named in the previous paragraph, different methods have been tested to attach corals (e.g. cable ties, epoxy resins, hydrostatic cement) (Forrester et al. 2011; Williams and Miller 2010), as well as different substrata (e.g. plastic trays, dead coral, rock, hanging ropes, plastic pins, etc.) (Levy et al. 2010; Rinkevich 2008). Rope nursery models that allow coral growth in all directions due to the good water flux and less encrusted organisms are being considered as one of the cheapest and least time-consuming methods, due to less fouling cleaning (Levy et al. 2010; O’Donnell et al. 2017). However, this method is an initial phase to grow corals faster, with the colonies latter having to be translocated to their final destination and survive the critical step of attaching to the reef. Failure of corals to self-attach to the reef is still one of the principal causes of death after translocation (Bongiorni et al. 2011). Improvements around this topic might solve a coral reef restoration bottleneck and help alleviate reef degradation at local scales (Rinkevich 2008).
Several *in situ* experiments have tested the effects of the initial fragment size on the success of culturing corals. A mix of different results has been obtained suggesting species-specific effects. For example, for the branching coral *Madracis mirabilis* (Duchassaing & Michelotti, 1860) larger fragment size does not improve survivorship (Bruno 1998). However, for *Porites lobata* Dana, 1846, *P. compressa* Dana, 1846 and *Acropora palmata* (Lamarck, 1816), larger fragments showed better growth and survival (Forrester *et al.* 2014; Forsman *et al.* 2006).

Another interesting *in situ* study, tested the effect of underwater electrodes to increase mineral accretion on nubbins of *Porites cylindrical* Dana, 1846, and found it to be an effective method to improve growth rate, specifically during the mineral accretion phase (around two months of age), and could be effective long-term at improving initial survival rates (Sabater and Yap 2002; 2004).

### 1.3.2 Coral aquaculture *ex situ*

The culture of corals *ex situ* involves some form of aquarium with seawater pumping through the culture system with or without recirculation (Borneman 2001). Currently, more than 100 species of coral are reproduced *ex situ*, and this number is rising (Borneman and Lowrie 2001). One of the great advantages of *ex situ* cultures is the opportunity to artificially control conditions to maximize coral production (Barton *et al.* 2017; Borneman and Lowrie 2001; Leal *et al.* 2016b). The reduction in undesirable environmental conditions, such as algal overgrowth and predation has allowed a significant improvement in survival rate, which can be more than 85% greater than *in situ* methods (Petersen *et al.* 2008).

Fragmentation is also the most common *ex situ* coral production method (Borneman and Lowrie 2001), and several techniques for attaching coral fragments have been developed (Arvedlund *et al.* 2003; Williams and Miller 2010). Usually, fragments placed horizontally can grow faster because they produce more than one vertical point of growth, whereas cuttings fixed vertically usually develop only one growth point and the calcium deposition is significantly reduced (Arvedlund *et al.* 2003). A detailed and successful protocol to produce coral nubbins is available for *Pocillopora damicornis* and *Stylophora pistillata* Esper, 1797 (see Shafir *et al.* 2006b), and coral clones originated from a single polyp have already been cultured for *S. pistillata* (Shafir *et al.* 2001). Other experiments that used aluminium plates to attach fragments of *Millepora dichotoma* Forsskål, 1775 showed highly accelerated growth after six weeks, suggesting that this substrate can improve skeletal bio-mineralization (Vago *et al.* 2001).
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Settlement of larvae at *ex situ* locations is a new approach to enhance survival and increases the genetic diversity of coral reefs (Guest *et al.* 2014; Petersen *et al.* 2005b; Petersen *et al.* 2008). This methodology might involve the transportation of mature colonies (gravid and close to spawning) to *ex situ* locations, then waiting for spawning, fertilization and settlement of the larvae to populate the new habitat (Guest *et al.* 2014). Additionally, due to the potential for dilution of gametes reducing fertilisation success and the dispersal of larvae in the water column, dela Cruz and Harrison (2017) have successfully employed the use of enclosures to release larvae that are ready to settle onto concentrated sections of the reef. However, the long-term nature and high costs of these methodologies constrain their application for large scale coral reef restoration (Guest *et al.* 2014). Some attempts have shown positive short-term outcomes, while others indicate that this is not an effective long-term alternative for reef restoration (Edwards *et al.* 2015).

Obtaining a large amount of genetically identical and uniformly sized fragments from *ex situ* cultures can be a great advantage for certain scientific experiments (e.g. to obtain identical replicates and test for colony effects) (Forsman *et al.* 2012). Furthermore, a wide range of research around ecological and eco-toxicological questions can be addressed using “multi-clone” cultivation (Hayashi *et al.* 2013). Additionally, it has been suggested that coral aquaculture *ex situ* is more favourable than *in situ* to supply natural products for drug discovery, as culture parameters can be manipulated using a selected controlled stressor to trigger mucus production and metabolites that can be harvested without destroying the coral host (Leal *et al.* 2013). More recently, selective breeding of resistant colonies to genetically enhance corals, has been proposed to help corals cope with climate change (van Oppen *et al.* 2015).

The potential for *ex situ* coral aquaculture systems was demonstrated by Shafir *et al.* (2001), who compared the survivorship *in situ* and *ex situ* of 300 nubbins of *S. pistillata*. After 103 days, 60% of the *ex situ* nubbins survived compared to no survival at the *in situ* location. Other important results obtained from their experiments showed that even species that do not fragment naturally, can survive when they are fragmented artificially (Shafir *et al.* 2001).

### 1.4 Feeding: autotrophy and heterotrophy

Traditionally, corals were considered as strict photoautotrophs that obtained fixed carbon through their endosymbiont algae (Veron 2000). However, many species (> 700) have no endosymbiotic algae and rely heavily on heterotrophy (Cairns 2004; Rogers 2004). Furthermore, corals with endosymbiont algae are also able to obtain carbon,
nitrogen, phosphorous and amino acids heterotrophically by consuming plankton, dissolved organic compounds and suspended particles (Ferrier-Pagès *et al.* 2011; Houlbrèque *et al.* 2003). To feed, the coral polyps extend their tentacles, usually during the night to catch prey (Borneman 2001; Porter 1974). It has been estimated that 35 to 60% of the metabolic requirements of corals comes from heterotrophy (Falkowski *et al.* 1993; Houlbrèque and Ferrier-Pagès 2009).

Coral aquaculture feeding experiments have demonstrated that when the corals species *Favia fragum* (Esper 1795) and *Acropora tenuis* (Dana 1846) were fed with either *Artemia salina* nauplii (brine shrimp), the pelagic diatom (*Phaeodactylum tricornutum*) and/or Nori Micro (a commercial dry food for invertebrates), they grew 5 to 8 times larger during the experimental period, than those without any food (Petersen *et al.* 2008). Because of the positive response of corals to this type of food, it was suggested that *Artemia* may be an appropriate substitute for wild zooplankton in aquaculture (Petersen *et al.* 2008). Furthermore, recruits of *Pocillopora damicornis* are able to feed on *Artemia* two days after settlement, with similar ingestion rates as adults (Toh *et al.* 2013b). It is likely that juveniles do not store extra energy acquired from feeding and use it directly to enhance their growth (Drenkard *et al.* 2013).

Plasticity between autotrophy and heterotrophy in corals is a strategy to maintain carbon supply during environmental stress (Forsman *et al.* 2012). For example, corals relying only on photosynthesis can survive during a bleaching event as long as their energetic reserves remain (Rodrigues and Grottoli 2007). The photosynthetic process does not provide enough of the essential organic components that are supplied by feeding (Osinga *et al.* 2011). Species capable of swapping from autotrophy to heterotrophy are able to recuperate faster from high temperature impacts (Grottoli *et al.* 2006; Rodrigues and Grottoli 2007), because more than 35% of nutrients obtained by bleached corals come from heterotrophy (Houlbrèque and Ferrier-Pagès 2009). When *Acropora cervicornis* (Lamarck, 1816) is maintained under high CO$_2$ environments, the species is capable of raising its feeding rate, and its stored lipids can alleviate any decreases in growth, which often occurs in unfed corals (Towle *et al.* 2015). *Montipora capitata* Dana, 1846 also increased feeding rates when exposed to high temperatures (Rodrigues and Grottoli 2007). Furthermore, larvae of *Favia fragum* subjected to high and low experimental CO$_2$ concentrations show significant growth increases when fed with *Artemia* nauplii relative to unfed larvae, regardless of CO$_2$ conditions (Drenkard *et al.* 2013).
Although there is still debate about the relative importance of heterotrophy versus autotrophy as mechanisms for energy acquisition in corals, the general consensus is that the relative importance of each process depends on species, light availability, depth, plankton abundance and stressors (Houlbrèque and Ferrier-Pagès 2009; Houlbrèque et al. 2003; Palardy et al. 2005; Palardy et al. 2006). For aquaculture purposes, there is a lack of information related to the concentration of food required to achieve maximum feeding rates of corals, the best time of the day for feeding (night or daylight) and maximum food size per coral species.

Lipids represent the main source of coral energy reserves (Rodrigues et al. 2008). Lipids are structurally important components of cell membranes and provide precursors for a range of vitamins and hormones. They are composed of fatty acid chains that vary in the degree of saturation (or number of double bonds) (Voet and Voet, 201). These reserves are important in minimizing the impacts of stressors in nature such as predators, diseases, strong currents, sediment, rising temperatures, as well as stressors in aquaculture, such as fragmentation processes, transportation and changing water conditions. However corals, like all other animals, are unable to biosynthesize some polyunsaturated fatty acids. These are regarded as essential fatty acids and must be obtained via the diet. Although the provision of lipid enrichment diets in other types of aquaculture is very common (e.g. fish and crustaceans), there is a lack of literature regarding the effect of the inclusion of lipid enrichments in the diet of corals.

1.5 Growth process, optimizing and measuring coral growth

The growth rate of coral species is variable. Fast growers, showing growth rates of 50 to 150 mm year$^{-1}$, usually have a branching shape (e.g. Acroporidae and Pocilloporidae), while slow growing corals, exhibiting growth rates of 5 to 10 mm year$^{-1}$, have massive shapes (e.g. Montastraea and Porites) (Buddemeier and Kinzie 1976; Dullo 2005). The major processes that influence coral growth are photosynthesis, heterotrophic feeding and calcification (Osinga et al. 2011). Other factors can also contribute, such as water movement and depth (Osinga et al. 2011), with coral growth rate decreasing with depth (Marubini et al. 2001). Furthermore, low pH can limit coral growth, through impacts on calcification, metabolism and symbionts (Maier et al. 2012; Tambutté et al. 2011). Poor calcification can also be attributed to reduced concentrations of aragonite (Marubini et al. 2001). Latitude seems to affect growth rates with a decrease towards high latitudes due to lower light intensity (Dullo 2005). However, every species has different requirements to achieve optimal growth (Forsman et al. 2012).
A thorough understanding of coral growth is essential for maximising coral aquaculture production. Overall, there are three typical ways to measure coral growth: (1) sacrificing the colony and measuring increments in calcification; (2) estimating surface increments; and (3) directly measuring increments in weight or length.

1.5.1 Measuring increments in calcification of corals

This technique uses tagging dyes, such as calcein, alizarin, oxytetracycline or alizarin complexone, to mark the coral skeletons. The coral is then sacrificed and sliced to examine the skeletal increments from the dye mark (Anderson et al. 2015; Holcomb et al. 2013; Kikuchi et al. 2013). However, this methodology is inaccurate in estimating growth rate in branching morphotypes (e.g. Acropora spp.) because in these corals new and old growth bands are not easily distinguishable from each other (Crabbe et al. 2008). Another available technique involves collecting a coral core, cutting a thick cross-section through the skeleton, and using radiography to count the annual growth rings (Guzmán et al. 1994).

These methods have advantages for research scenarios. For instance, the dye uptake method permits marking a point in time along the skeleton. Afterwards, the growth bands can be measured to calculate growth in time (Sabater et al. 2002; Holcomb et al. 2013), or to identify banding patterns that could be useful to detect environmental responses (Kikuchi et al. 2013) after a treatment or event (e.g. heavy metal exposure) allowing comparisons before and after the dye mark.

1.5.2 Surface estimation of corals

Surface estimation methods often have limited accuracy, are time consuming, and cannot be practically used for in situ measurements at a reef scale (Courtney et al. 2007; Holmes 2008). Under aquaculture conditions, these methods are not used because most of them require the sacrificing of the colony to obtain the measurement. However, there are several options to estimate coral surface that are commonly used to estimate growth rate and are considered useful to compare data among different corals. Among the most established methods to estimate surface coral area are: calculation of projected area (Odum and Odum 1955), surface index (SI) (Dahl 1973), dye uptake (Hoegh-Guldberg 1988), wax dipping (Stimson and Kinzie 1991), x-ray CT scanning (Veal et al. 2010), laser measurements (Vago et al. 1997), aluminium foil (largely used for boulder-shape colonies, such as Porites spp.) (Marsh 1970), latex coverage (Meyer and Schultz 1985),
photogrammetry and 3D modelling (Bythell et al. 2001; Courtney et al. 2007), combined 2D projected area and 3D colony surface area (Holmes 2008), 3D reconstruction using videos (Cocito et al. 2003), computer tomography and 3D surface area reconstruction (Naumann et al. 2009) and determination of aerial ratio increase using photography (Tanaka et al. 2013). Every method listed above must be selected depending on the morphotype and the budget availability.

1.5.3 Measuring increments in weight or length of live corals

Directly measuring increments in weight or length is the preferred option under aquaculture conditions, because these methods are relatively simple to carry out, less time consuming, cheaper than the previous two approaches and do not require the corals to be sacrificed. For this reason, they can be used on a regular basis. However, as with the previous options, there are a large variety of methods that can be used (each one with advantages and disadvantages). Thus the selection of which method is the most adequate for each coral species or project should be carefully considered according to the specific project objectives and potential for artefacts. Some techniques measure coral growth increments through buoyant weight (Bak 1973; Davies 1989), drip-dry weight or wet weight (Schlacher et al. 2007), while others measure length or volume (Herler and Dirnwöber 2011; Mercado-Molina et al. 2015). Of these, the drip-dry weight is likely to introduce more error than the buoyant weight or volume due to variability in the amount of water retained on the coral surface, whereas measure of the length is not suitable for boulder-shaped corals (e.g. Porites), but useful to obtain linear growth of branching corals.

There are several options and commonly used methods to measure coral growth rate: basal proportion (Green et al. 2010), daily linear extension (Mercado-Molina et al. 2014), linear increment in height (Gomez et al. 2014), geometric mean diameter (Clark and Edwards 1995), colony radius (Gomez et al. 2014) and ecological volume index, assuming a cylinder shape or total space occupied by the colony in the ecosystem (Rinkevich and Loya 1983). A brief description of each technique and related measurement formulas are included in Appendix 1.

Ultimately, every method has advantages and disadvantages. However, a combination of buoyant weight and the linear extension method is a practical approach because higher skeletal density could increase the weight without increasing height (Marubini and Thake 1999). The selection and implementation of methods to estimate coral growth should consider the costs involved and coral shape. In general, the growth
of plate-shaped corals can be easily obtained using a measurement of surface area or linear extension rate, whereas the boulder-shaped species can be more easily measured using surface area, and branch-shaped species by using wet weight and buoyant biomass (Osinga et al. 2011; Turner et al. 2015).

1.6 Potential applications of coral aquaculture

The three major applications for coral aquaculture are: i) coral reef restoration, ii) production of corals for the aquarium trade; and iii) production of corals for the drug trade and for research supply. Below is a summary of these applications.

1.6.1 Coral reef restoration

Since 1996, corals have been transplanted to restore damaged reefs (Rinkevich 2000). Ecological restoration techniques are constantly improving and are being commonly used to enhance Marine Protected Areas (Rinkevich 2014). However, it has been emphasized that first, the causes for the demise of a reef should be identified and remediated before any restoration activity begins (Mumby and Steneck 2008).

Coral reefs can recover reasonably fast when environmental stress is released (Gilmour et al. 2013). The predicted scenarios of reef demise caused by climate change present new challenges and require restoration strategies that focus on corals that can cope with future warmer environments (Rinkevich 2014). For some species, such as Acropora cervicornis, natural recovery after a decline caused by storms could be impossible due to low reattachment and low survival, which suggests that human intervention may be necessary (Mercado-Molina et al. 2015). Nonetheless, transplantation should be considered as a last resort, but in those cases where it is justified, restoration should focus on the addition of massive coral species first because they often recruit slowly, instead of short-term and short-lived species that recruit well (Edwards and Clark 1998).

Traditionally, reef restoration projects have centred on the transplantation of entire colonies or fragments from nearby reefs. However, *ex situ* transportation of coral larvae, at densities lower than 4 ml⁻¹, has been successfully used in Acropora tenuis, A. digitifera (Dana 1846) and Diploria strigosa (Dana 1846) (Petersen et al. 2005a). Additionally, it has been observed that restoration projects using patches of corals with high cover can be more suitable than patches with low cover of dispersed corals (Mumby and Steneck 2008). In this sense, several techniques have been developed and are available for restoration projects, each with its own advantages and disadvantages (Omori et al. 2006; Shafir et al. 2006a; Shaish et al. 2008). Recent studies recommend rope
nurseries over table bed nurseries; however, details regarding nursery structure, materials, sites, depth, fouling control methods, period of nursery, fragment size, genotypes, and coral source are still debated (Levy et al. 2010). For instance, an approach has been proposed whereby floating nurseries act as a hub for larval dispersion, which can be relocated during the reproduction period to enhance recruitment at targeted reefs (Amar and Rinkevich 2007).

There is debate about the use of coral sexual or asexual reproduction for reef restoration projects. As explained in a previous section (see 1.4.2 Coral reproduction) each reproductive method has advantages and disadvantages. Both methods have limitations on what scale they could be implemented. In any case, these methods do not necessarily need to exclude each other and a combinations of both can be used to better address the different anthropogenic impacts on the reefs. However, due to the current fast decline of coral reefs, other solutions such as assisted adaptation need to be considered (e.g. coral epigenetic programming, selective Symbiodinium process, selective breeding and assisted gene flow) (van Oppen et al. 2017).

1.6.2 Aquarium trade of corals

Historically, the aquarium market has been dominated by freshwater species and only recently have marine “mini-reef” systems become popular (Larking and Degner 2001). The first commercial coral propagation company started in the 1960s in Michigan, North America (Delbeek 2001). However, the inadequacy of aquaculture broodstock has meant that the aquarium trade is still dependent on the harvesting of natural corals (Rinkevich 2008; Rinkevich 2014).

In 2001, about 131 species of coral were being cultivated by hobbyists, 28 species by scientists, and 55 species by commercial breeders (Moe 2003). Nevertheless, the majority of corals sold in the aquarium trade are sourced from coral reefs from tropical developing countries (Barton et al. 2017; Moe 2001). Product certification, traceability and resource management, are identified as top priorities to improve the marine aquarium trade and commercial aquaculture (Murray and Watson 2014). The marketplace still needs to increase its acceptance of cultured over wild-caught specimens, and to embrace higher prices in order to obtain many advantages of cultured products, such as a more reliable supply, increased quality and survival, and consistent size and coloration (Corbin et al. 2003).
1.6.3 Drug trade and research supply of corals

Marine life offers potential for drug discovery and corals have already been recognized as a good source of bioactive compounds (Leal et al. 2013; Leal et al. 2016a). In the past two decades, ~3000 natural products have been discovered from corals (Rocha et al. 2011). The demand for new drugs from corals is always increasing, but the large quantities of coral biomass required for volume drug production is an issue in relation to the sustainability of marine ecosystems (Leal et al. 2013).

Corals bio-products have been investigated for cancer, blood disorder, skincare treatments, UV screens and many other applications (Calfo 2001). Aquaculture could stabilize the chemical composition of corals required for drug research by raising corals in a homogeneous environment. It could also optimize coral metabolite production and coral tissue growth through selective breeding (Leal et al. 2013). Increases in the need for corals for research purposes has been emphasized by Shafir et al. (2006b), and physiological studies have stressed the necessity to obtain genetically identical living material. Nonetheless, the focus in drug discovery research for coral aquaculture should be directed to increasing the production of the soft tissue around the coral skeleton, necessary to support the drug development experiments (Leal et al. 2013). Furthermore, recent research has demonstrated that coral matrix proteins can be used to accelerate human bone regeneration (Green et al. 2017).

1.7 Current limitations in commercial coral aquaculture

Improvements in coral aquaculture protocols are necessary to achieve economical sustainability (Wijgerde et al. 2012). Moreover, many more species of corals are yet to be investigated for culture optimisation, and new combinations of culture parameters still need to be verified (Arvedlund et al. 2003). A major challenge for intensive aquaculture is to find the optimal nutrition to achieve the best survival and growth (van Os et al. 2012). In addition, testing different combinations of growth factors provides almost infinite possibilities of experiments that can be undertaken to improve knowledge in this area (Arvedlund et al. 2003).

In relation to climate change and reef restoration projects, world-wide studies are needed to establish the tolerance and resilience characteristics of corals to thermal stress predictions, and to understand the rate of natural adaptation (Logan et al. 2014). The understanding of response mechanisms of corals to stress and acclimatization is considered one of the priority areas for research (Buddemeier and Smith 1999). Further research should include the culture of corals under predicted future environmental conditions.
conditions as well as investigation of the potential resilience of deeper water corals and their adaptability to aquaculture and as a source for reseeding reefs. Thus, the selection and breeding of colonies that survive those conditions could help to accelerate the thermal tolerance in some species (van Oppen et al. 2015).

1.8 Research aims

The present thesis explores new methods for optimizing the production of tropical coral species, with the overall objective of improving coral aquaculture protocols. The specific aims of this thesis are to:

(i) optimise heterotrophic feeding of corals under ex situ aquaculture conditions;
(ii) develop a new technique to increase polyp production using coral aquaculture waste fragments;
(iii) improve coral self-attachment to the substratum; and
(iv) test if a lipid-enriched diet enhances growth and coral resilience under normal and high temperatures.

The thesis comprises a series of connected but stand-alone chapters addressing the four aims above. It is presented as manuscripts that are published. Given that the experiments were done in the same aquarium system, there is some repetition among the methods. Where possible, this has been minimised. A brief description of Chapters 2 to 6 follows.

Chapter 2 examines the feeding rates of the three different coral species (Acropora millepora (Ehrenberg 1834), Hydnophora rigida (Dana 1846) and Duncanopsammia axifuga (Milne Edwards and Haime 1848)) used in all the subsequent chapters. Six different concentrations of Artemia were used to estimate the maximum feeding rates and the necessary concentration to achieve those maximum feeding rates, as well as the saturation point where corals did not increase their feeding. The optimum concentrations of food obtained in this chapter were used as reference in all subsequent chapters. This chapter has been published as:


Chapter 3 details a 100 day manipulative experiment to investigate the survival and growth rate of the stony coral Duncanopsammia axifuga. It specifically tested whether ‘waste fragments’ or trunks initially fixed without polyps can produce new polyps, in comparison to trunks initially fixed with one polyp. The influence of fixing orientation
(right way up or upside-down) and the effect of three different diet regimes (unfed, normal *Artemia* and lipid-enriched *Artemia*) on the coral variables responses (i.e. buoyant weight, chlorophyll, algal symbiont density, colour and maximum quantum yield) was also tested. This chapter has been published as:


**Chapter 4** reports whether the ‘upside-down’ method of fixing coral fragments can also accelerate the self-attachment of a branching coral species to the substratum, in order to increase the survival rate of translocated corals in restoration projects. The effects of three different diets (unfed, normal *Artemia* and lipid-enriched *Artemia*) over several growth variables responses (i.e. buoyant weight, basal growth, height increment) and other coral health indicators such as chlorophyll, algal symbiont density, colour and maximum quantum yield) were also examined. This chapter has been published as:


**Chapter 5** investigates whether corals can improve their overall health condition at normal temperatures (26 °C) and survive a bleaching event of 14 days at 32 °C, when fed a lipid-enriched diet with a high proportion of polyunsaturated fatty acids (particularly, omega-3 fatty acids, docosahexaenoic and eicosapentaenoic). The results were compared against unfed corals and corals fed with an unenriched diet. This chapter has been published as:


**Chapter 6** provides a summary of the findings of this thesis and outlines recommendations for further research necessary to build on these results. It is hoped this thesis will provide useful new tools for coral aquaculture, thereby contributing to a reduction in wild coral collection and improving the success of coral reef restoration projects.
Chapter 2 - Optimizing heterotrophic feeding rates of three commercially important scleractinian corals

This chapter has been modified, to comply with the University thesis guidelines, from the following published paper:

ABSTRACT

Heterotrophy plays an important role in improving coral growth rate in aquaculture. While a lack of sufficient food will reduce growth and delay production timelines, excessive food deteriorates water quality and represents an additional production cost without benefit. Currently, there is limited information about the maximum feeding rates of corals. Using the curvilinear Michaelis-Menten model with six different concentrations of *Artemia* during day and night feeding, I estimated the maximum feeding rates for *Acropora millepora* (4.6 ind cm$^{-2}$ h$^{-1}$), *Hydnophora rigida* (20.4 ind cm$^{-2}$ h$^{-1}$) and *Duncanopsammia axifuga* (22.8 ind polyp$^{-1}$ h$^{-1}$), and the necessary concentrations of food to reach the maximum levels of feeding. Overall, the three species increased their feeding rates with increasing *Artemia* densities until the concentration reached 120 ind ml$^{-1}$, after which there was no change in feeding rate. No significant differences were detected between day and night feeding rates for the three corals species. The concentration to reach the maximum *Artemia* feeding rates for these commercially important coral species was above 50 ind ml$^{-1}$.

2.1 Introduction

Aquaculture provides corals for reef restoration projects, the aquarium trade, drug discovery and research (Delbeek 2001; Leal *et al.* 2016a), as well as contributing to employment in coastal tropical developing countries (Ferse *et al.* 2012; Rinkevich 2015; Tadinanahary *et al.* 2017). There are at least 117 coral species that have been cultivated, out of over 300 described species (Rinkevich 2014; Veron 2000). However, there is limited available information related to coral feeding which is hampering efforts aimed at improving coral production (Hii *et al.* 2009; Wijgerde *et al.* 2011).

Heterotrophy has an important role in the energy budget of scleractinian corals because many nutrients that cannot be supplied by photosynthesis are acquired through feeding (Houlbrèque and Ferrier-Pagès 2009). As feeding also has a positive effect on tissue and skeletal coral growth (Ferrier-Pagès *et al.* 2003), one of the largest challenges in intensive coral aquaculture is to find the optimal nutrition to achieve the fastest growth rate (van Os *et al.* 2012). However, there are only a limited number of studies that have investigated coral feeding rates from an aquaculture perspective (van Os *et al.* 2012; Wijgerde *et al.* 2011), with most focusing on coral feeding in an ecological context (Anthony 1999; Houlbrèque *et al.* 2003; Treignier *et al.* 2008). Since corals often have species-specific conditions for optimal growth (Forsman *et al.* 2012), many coral species...
remain unexamined, and innovative combinations of different culture parameters require further experimental evaluation (Arvedlund et al. 2003).

As some corals feed at night and others during the day (Porter 1974; Borneman 2001), the quantitative determination of food demand from cultivated corals and the best time to feed them is desirable for cost-effective aquaculture (Lin et al. 2002). Long coral rearing periods makes it necessary to identify feeding regimes, times, and frequency, as well as duration of the feeding period, that maximize growth rate and survival, while minimising the financial burden of these additional processes (Toh et al. 2014). For that reason, it is useful to know the feeding satiation point, where corals stop feeding once they have captured a certain quantity of food (Lin et al. 2002). Food addition just below this coral feeding satiation point is recommended as a strategy to reduce food wastage and avoid water quality deterioration (Ali et al. 2010). Artemia is a valuable live food for aquaculture because it is palatable to a great variety of aquatic organisms (including freshwater species) and can be stored in a cyst form almost indefinitely (Dhont and Sorgeloos 2002). Artemia is, therefore, the most commonly used live food in aquaculture (Dhont and Sorgeloos 2002; Petersen et al. 2008). Corals are also often fed Artemia (Houlbrèque et al. 2003; Yii-Siang et al. 2009) because it enhances growth and survivorship in both ex situ nurseries and after being transplanted to reefs in restoration projects (Toh et al. 2014). However, as some coral species may have an upper prey-size limit due to their small polyp size (Houlbrèque and Ferrier-Pagès 2009), the acceptance of Artemia should be investigated for each coral species considered for cultivation.

Here, Artemia feeding rates were optimised for three commercially important coral scleractinian species: (1) Acropora millepora, a reef-building species that is one of the most studied scleractinian corals in the Great Barrier Reef, Australia, and it has commercial relevance to the aquarium trade and restoration projects (Bongiorni et al. 2011; Borneman and Lowrie 2001; Ramos-Silva et al. 2014); (2) Hydnophora rigida, a species with considerable demand for the aquarium trade and also used for restorations (Bongiorni et al. 2011); and (3) Duncanopsammia axifuga, a species with increasing demand in the aquarium trade (personal observation). In order to enhance ex situ aquaculture protocols, the main objective of this study was to provide the maximum feeding rates for these three scleractinian coral species, as well as to assess whether heterotrophic feeding rates would be greater at night than during the day.
### 2.2 Material and methods

This study was carried out at the National Marine Science Centre (NMSC), Southern Cross University, Coffs Harbour, Australia (30°16′3.24″S–153°8′16.26″E). The feeding experiments were done under natural daylight (12:00 pm) and at night (after 7:00 pm) in outdoor tanks. Seawater was pumped from the adjacent open-ocean. To avoid other sources of food influencing the experiment, the seawater was filtered using a sand gravel filter, a 50 μm filter, a cartridge particle filter (EMAU® model CF25), a zeolite media filter (JNS model FR-2E, Japan), an in- sump protein skimmer (JNS model SK-6, Japan), an activated carbon media filter (JNS model FR-2E, Japan) and all running via a pump (Doughboy model 72.543, 1HP, 18m3/h, UK). Salinity was maintained at 36 ± 0.5 ppm. Temperature was kept stable at 26 ± 0.5 °C, using a temperature controller (2100 l/h, EVO-F5, Australia).

Eight colonies of each studied species: *A. millepora*, *D. axifuga* and *H. rigida* (Fig. 2.1), were collected near Cairns on the Great Barrier Reef and purchased through Cairns Marine. Each colony was acclimatized for at least one month and fed twice a week with *Artemia*. Once acclimatized, each colony was fragmented into similar size pieces using a cordless Dremel® (model 8220, USA). Thirty five fragments per species were fixed to glass stoppers (Vinolok, Czech) using cyanoacrylate (super glue) (Gel control, Loctite®). All fragments were kept for an additional month until all injuries from the fragmentation process were healed. Fragments were pooled and randomly selected. Only corals that appeared to be healthy (polyp fully open, rich colour and full tissue coverage) were used.

One day before commencing the feeding trials, 10 g of *Artemia salina* cysts (“AAA” grade GSL Artemia Cyst, INVE, Belgium) were incubated in a 10 l well-aerated hatching cone with direct light and water at ~ 28 °C and salinity at ~ 36.5 ppm. To avoid any unhatched cysts getting into the culture, a magnetised cyst collector tube was used (Sep-Art™, INVE, Belgium). The nauplii were maintained at a density of 400 individuals ml⁻¹ (ind ml⁻¹) in 8 l of filtered seawater; afterwards, the water volume was reduced to increase the concentration of the *Artemia* stock (~ 2000 ind ml⁻¹). Then, different aliquots of the concentrated stock and appropriate amounts of seawater were added to the vials to obtain the desired concentrations (i.e. 2, 5, 20, 50, 120 and 250 ind ml⁻¹). The same concentrations without corals were used as procedure controls.

Three hours before commencing the feeding, five coral replicates for each *Artemia* concentration were transferred into independent replicate vials of 250 ml. Corals in each vial were randomly located in a recirculating water bath attached to the main tank to avoid temperature changes. Homogeneous water movement or agitation in each vial was
generated by using independent air hoses connected to glass pipettes (diameter 1 ml) reaching the bottom of the vials. Water overflow was avoided during the feeding hour.

The different volumes of concentrated *Artemia* were pipetted into the vials. Each coral was incubated with the respective *Artemia* concentration for 1 hour. After this time, five replicates of 1 ml were taken from each vial with an automatic pipette and placed in separate Eppendorf vials with 0.5 ml of 100 % Ethanol. All samples (n = 5 per concentration) were counted using a stereoscopic microscope during the following week. Feeding rates were calculated using a modification of the formula proposed by Hii *et al.* (2009).

Feeding rate = (Initial - Final *Artemia* concentration) / Surface area or # of polyps × Time

**Figure 2.1** Coral species used in the present study, with details on polyp size. From top to bottom: *Acropora millepora*, *Hydnophora rigida* and *Duncanopsammia axifuga*.
The choice of normalization methods for each coral species was based on polyp size. *A. millepora* and *H. rigida* are small polyp species suited for the surface area method, whereas *D. axifuga* has large polyps easier to normalize with the number of polyps.

Using the Enzyme Kinetics (Michaelis-Menten model) application of SigmaPlot 13 (Systat software, San Jose, CA), the maximum feeding rates (Vmax; ind cm\(^2\) h\(^{-1}\)) and *Artemia* densities at which half-saturation occurs (Km; ind / ml) were calculated based on the feeding rates of corals exposed to six different *Artemia* concentrations.

### 2.3 Results

Based on the curvilinear Michaelis-Menten model, maximum feeding rates (Vmax) and half-saturation constant (Km) at which corals reach those velocities were calculated for *A. millepora*, *H. rigida* and *D. axifuga* (Table 2.1, Fig. 2.2). *D. axifuga* showed the highest feeding rates, whereas *A. millepora* presented the lowest. *D. axifuga* and *H. rigida* showed relatively similar *Artemia* concentrations to reach half of the feeding saturation or Km values.

Confidence intervals for Km estimations were wide for all species, but relatively narrow for Vmax estimations; however, non-linear regressions for all species during both day and night were significant and with an R > 0.79.

For each of the three coral species, feeding rates constantly increased with higher *Artemia* concentrations up to 120 art ml\(^{-1}\). After this concentration, the coral feeding rates reached saturation and stopped increasing, or even decreased in the case of *H. rigida*. The overall feeding rates did not differ significantly between day and night and there were no significant interactions between time of day and *Artemia* concentration (Table 2.2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Time</th>
<th>Vmax</th>
<th>IC</th>
<th>Km</th>
<th>IC</th>
<th>LC</th>
<th>R(^2)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acropora millepora</em></td>
<td>day</td>
<td>2.80</td>
<td>1.79</td>
<td>3.80</td>
<td>50.58</td>
<td>-3.07</td>
<td>104.23</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.57</td>
<td>1.00</td>
<td>8.14</td>
<td>189.20</td>
<td>-86.05</td>
<td>464.36</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>night</td>
<td>4.57</td>
<td>1.00</td>
<td>8.14</td>
<td>189.20</td>
<td>-86.05</td>
<td>464.36</td>
<td>0.80</td>
</tr>
<tr>
<td><em>Hydnophora rigida</em></td>
<td>day</td>
<td>20.84</td>
<td>9.25</td>
<td>32.40</td>
<td>85.80</td>
<td>-31.71</td>
<td>203.31</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.35</td>
<td>8.33</td>
<td>30.40</td>
<td>77.01</td>
<td>-35.20</td>
<td>189.22</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>night</td>
<td>19.35</td>
<td>8.33</td>
<td>30.40</td>
<td>77.01</td>
<td>-35.20</td>
<td>189.22</td>
<td>0.63</td>
</tr>
<tr>
<td><em>D. axifuga</em></td>
<td>day</td>
<td>20.25</td>
<td>9.14</td>
<td>31.40</td>
<td>96.22</td>
<td>-23.34</td>
<td>215.79</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.81</td>
<td>15.6</td>
<td>30.00</td>
<td>94.70</td>
<td>26.87</td>
<td>162.54</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 2.1 Summary of the curvilinear regression Michaelis-Menten model of the three coral species. Vmax = maximum feeding rate, Km = half-saturation constant. IC = interval of confidence.
Table 2.2 Summary of permutational analysis of variance results for three coral species fed with six different *Artemia* (Art.) concentrations during daytime and night time (day/night). Bold = significant (at \( p < 0.05 \)).

<table>
<thead>
<tr>
<th>Art. concentration</th>
<th>Pseudo-F</th>
<th>( p(\text{perm}) )</th>
<th>Day/ Night</th>
<th>Pseudo-F</th>
<th>( p(\text{perm}) )</th>
<th>Art. vs. Day/ Night</th>
<th>Pseudo-F</th>
<th>( p(\text{perm}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acropora millepora</em></td>
<td>19.080</td>
<td><strong>0.0001</strong></td>
<td>3.6566</td>
<td>0.0628</td>
<td>0.7884</td>
<td>0.5754</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hydnophora rigida</em></td>
<td>12.785</td>
<td><strong>0.0001</strong></td>
<td>2.5338</td>
<td>0.1183</td>
<td>0.8625</td>
<td>0.5203</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. axifuga</em></td>
<td>34.634</td>
<td><strong>0.0001</strong></td>
<td>0.6369</td>
<td>0.4275</td>
<td>0.3758</td>
<td>0.8644</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant differences were detected across growing *Artemia* densities. For *H. rigida*, all concentrations were significantly different when compared among each other and a significant reduction in the feeding rate was observed after 120 ind ml\(^{-1}\). Whereas for *A. millepora* and *D. axifuga*, significant differences were detected across all the concentrations, except between 120 and 250 ind ml\(^{-1}\), indicating that a feeding saturation point was reached (Table 2.2, Fig. 2.3).

![Figure 2.2](image-url)

**Figure 2.2** Feeding rates as a function of *Artemia* concentration for three coral species during the day and the night. Data shows the mean ± standard error. Black lines show the best fit to the curvilinear Michaelis-Menten regression model and blue lines represent 95% confidence limits.
Figure 2.3 Comparisons of feeding rates of three different coral species under different concentrations of Artemia, as well as comparisons of feeding rates during the day and the night. Bars represent the means (+ SE) of the main effects, as there were no significant interaction between day/night time and Artemia concentrations. Significant groups (at $p < 0.05$), derived from pairwise post hoc tests, are indicated with different letters.
2.4 Discussion

Coral feeding rate is dependent on prey concentration (Ferrier-Pagès et al. 2010) and, as a consequence, it increases with prey availability until a maximum feeding rate is reached (Palardy et al. 2006). When corals are fed with higher concentrations than the half-saturation constant (Km), no further increase in the feeding rate is expected (Hii et al. 2009). Consequently, feeding above this rate results in Artemia not being eaten by corals, which not only reduces cost effectiveness, but also causes nitrogen and phosphorous to increase in the water column, negatively affecting growth and survival (Ali et al. 2010; Petersen et al. 2008). A deterioration of water quality is particularly problematic for closed recirculation systems, compared to flow through systems where seawater is constantly refreshed (Sheridan et al. 2013). To avoid such issues in aquaculture, and to maximize coral growth, this study determined maximum feeding rates for three commercially important species.

For each of the three coral species investigated, feeding rate increased with food concentrations, until a maximum feeding rate was observed, beyond which ingestion stayed constant (i.e. A. millepora and D. axifuga), followed by declining feeding rates beyond the saturated point (i.e. H. rigida). Such results were observed after 120 ind ml$^{-1}$ for all three coral species. Previous work with A. millepora, which used non decapsulated Artemia cysts to simulate sediment particles, did not find a saturation point for this species using a maximum concentration of 30 mg l$^{-1}$ (Anthony 1999). Another study using Artemia nauplii fed to A. millepora found a feeding rate of 0.13 ind polyp h$^{-1}$, using a low food concentration (0.3 ind ml$^{-1}$) and long feeding times (12h) (Kuanui et al. 2016). Other coral species, such as Galaxea fascicularis, have maximum feeding rates that exceed those of the three species in the present study (Hii et al. 2009), whereas similar results have been reported for Turbinaria reniformes and Stylophora pistillata with 20 and 27 ind polyp h$^{-1}$, respectively, although using 30 Artemia per polyp as the food concentration (Ferrier-Pagès et al. 2010). For the three coral species investigated, there are no other studies providing Artemia maximum feeding rate using six different concentrations of food.

Although all species of corals investigated here showed the same general patterns of feeding rate, there were species specific differences in rates of heterotrophic feeding, probably due to differences in polyp size among species, as well as variations in feeding strategies. The three coral species investigated fitted into two broad feeding strategies, as described by Lewis and Price (1975), with the small polyp species (such as A. millepora and H. rigida) feeding by combining tentacle capture and mucus entanglement, whereas
the large polyp species *D. axifuga* was observed only feeding by tentacle capture. Given this, the variation in feeding rates among coral species maybe due to feeding mechanisms, morphology, number of tentacles and polyps, and coelenteron sizes (Hii *et al.* 2009; Johnson and Sebens 1993). Nevertheless, it has been suggested that coral feeding effort and not necessarily polyp size or coral morphology, is a major factor in influencing species specific feeding rates (Palardy *et al.* 2005).

As water movement influences coral prey capture (Forsman *et al.* 2012; Sebens *et al.* 1997), current must be considered when interpreting results of coral feeding. In this study, an air hose was connected to a glass pipet that reached the bottom of the vials to generate water movement. This technique was an effective way to allow water and *Artemia* circulation throughout the vial, while maximizing the space and water volume available. Given the tight spatial constraints, this method was preferred to a bulk air-stone bubble diffuser frequently used in aquaculture systems.

The Km parameter in a coral feeding context is a direct reflection of the affinity or voracity of the coral towards certain prey, with species showing low Km values being much more voracious and capable of reaching the maximum feeding rate at lower concentrations of food, than those coral species showing higher Km values (Voet and Voet 2011). This requires consideration of cost-effective culturing of particular corals, as species that are strongly heterotrophic will grow faster and might produce up to eight times higher tissue growth and 30 % higher calcification rates that those with a lower feeding voracity or which are unfed (Ferrier-Pagès *et al.* 2003). However, the variation in Km among the three species suggests that some caution is required when selecting the appropriate concentration of food to reach the maximum feeding rate. For *A. millepora* and *H. rigida*, the estimated Km appeared to be high in relation to the calculated maximum feeding rates (Vmax) and might be explained by the observed mucus release, acting as an efficient trap to increase prey capture (Naumann *et al.* 2009). Nevertheless, mucus trapping could affect the accuracy of the calculated maximum feeding rates, as not all *Artermia* trapped in the mucus are consumed by the corals. Consequently, the maximum feeding rate could sometimes be overestimated for coral species using mucus trapping.

Wild corals tend to feed at night when polyps are fully expanded (Borneman 2001; Porter 1974), which would imply additional cost related to employing aquarists to feed corals outside of normal business hours. However, no significant differences were found between the day and night feeding rates in the three coral species, and similar results have been previously observed for other cultivated coral species (Hii *et al.* 2009; Kuanui *et al.*
Corals most likely feed at night in natural environments because zooplankton densities are highest during the night (Heidelberg et al. 2004; Nakajima et al. 2008). Nevertheless, feeding activation occurs in the presence of food chemical signals (Lehman and Porter 1973) and corals can be ‘trained’ to feed during the day, changing their feeding behaviour depending on the availability of food (Borneman 2001). The present results corroborate these ascertain and are an important consideration for aquaculture, as night feeding may be associated with additional production costs.

This Chapter estimated the maximum feeding rates of three commercially important species of coral, as well as the Artemia concentrations at which half saturation point is reached. The results showed that corals display similar feeding rates during the day and night. This information can be used to enhance coral aquaculture protocols to obtain optimum growth rates while avoiding water quality issues associated with overfeeding, as well as reduce additional costs associated with nocturnal feeding of corals.
Chapter 3 - A new technique to increase polyp production in stony coral aquaculture using waste fragments without polyps

This chapter has been modified, to comply with the University thesis guidelines, from the following published paper:


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ABSTRACT

The wild harvest of corals for the aquarium trade has had negative impacts on coral reef ecosystems. Although this problem can be directly addressed by increased availability of cultured corals, improvements to current aquaculture techniques are needed to increase coral production. Here, a manipulative experiment was carried using the large polyp reef-building scleractinian coral *Duncanopsammia axifuga* to test whether the presence or absence of polyps, the orientation of the fragment's trunk (upside-down or right way up), or heterotrophic diet (unfed, normal *Artemia* and lipid-enriched *Artemia*) influences polyp production, growth, survival, and overall health of the coral. There was an 85% survivorship of fragments across all experimental treatments regardless of trunk orientation. *D. axifuga* produced the highest number of new polyps from fragments initially fixed without polyps, with diet having no effect on polyp production. Mean ± SE polyp production was recorded for trunks initially without polyps, right way up and fed with enriched *Artemia* (3.5 ± 1.3), followed by trunks initially without polyp that were upside-down and unfed (3.8 ± 0.7). Conversely, normal and lipid-enriched *Artemia* diets significantly enhanced growth and polyp production in fragments that were initially fixed with one polyp. Overall, it is concluded that *D. axifuga* is suitable for coral aquaculture purposes due to high survival rates and also because polyp production was successful from what was previously considered waste fragments (i.e. without polyps). Further work is required to determine whether these aquaculture techniques enhance polyp production in other coral species with similar morph types used in commercial aquaculture.

3.1 Introduction

Coral aquaculture is a rapidly expanding industry (Osinga *et al*. 2011; Rhyne *et al*. 2012), with the aquarium trade growing from 250,000 live corals in 1991 (Wells and Hanna 1992) to around 1.5 million live corals per year (2010–2014) (CITES 2016). The sector has increased production to supply the aquarium market, pharmaceutical industry and reef restoration enterprises, as well as to reduce wild collection on already declining natural coral reefs (Leal *et al*. 2013; Todinanahary *et al*. 2017). Nonetheless, wild catches still represent over 78% of the source of corals for the aquarium trade (CITES 2016), which provides a clear incentive for greater coral aquaculture production and improved culturing techniques (van Os *et al*. 2012).

Coral fragmentation is an important procedure for coral aquaculture (Leal *et al*. 2016a). This process also naturally occurs in two different ways: i) by wave action (Denny
et al. 1985; Highsmith 1982); and ii) as a ‘programmed fragmentation’ or asexual reproduction mode that also promotes dispersal (Tunnicliffe 1981). Natural fragmentation is harnessed in coral restoration projects by collecting the fragments and reattaching them to enhance survival (Williams and Miller 2010). However, fragments also have the potential to survive independently (Smith and Hughes 1999). After a substantial wave event, most natural coral fragments are often loosely attached to the substratum or stuck in reef crevices (Bruno 1998); within this context, it can be assumed that some of these fragments will get trapped upside-down. It is, however, not clear whether such fragments survive or indeed grow polyps faster to compensate for reduced light availability to existing polyps.

Coral survivorship and growth are affected by many biotic and abiotic factors (Bell and Gervi 1999; Shafir et al. 2001). As ideal culture conditions of many coral species have not been determined (Bell and Gervi 1999; Shafir et al. 2001), there is, therefore, scope to evaluate combinations of different culture parameters for several coral species in order to improve aquaculture production efficiency (Arvedlund et al. 2003). In particular, different feeding regimes often result in improved coral growth because corals are able to assimilate diverse nutritional sources from particulate matter to macrozooplankton (Anthony 1999; Houbrèque and Ferrier-Pagès 2009), but as coral heterotrophy can be species specific (Leal et al. 2014), further research is required.

Duncanopsammia axifuga (Milne Edwards and Haime, 1848) is a large polyp species catalogued by the International Union for Conservation of Nature (IUCN) Red List as a Near Threatened species (Hoeksema et al. 2008). It is a popular aquarium species, with a relatively high monetary value in the trade. However, there are no published studies evaluating methods for optimising its aquaculture production. Here, the survival and growth rate of the stony coral D. axifuga was investigated, specifically testing whether ‘waste fragments’ or trunks initially fixed without polyps can produce new polyps, in comparison to trunks initially fixed with one polyp. I also tested if the fixing orientation (right way up or upside-down) has an effect on the growth and survival of the coral fragments, as well as the impacts of three different diet regimes (unfed, normal Artemia and lipid-enriched Artemia) on the coral variables responses (i.e. chlorophyll, algal symbiont density, colour and maximum quantum yield).
3.2 Material and methods

3.2.1 Corals fragmentation and experimental setup

Eight healthy (i.e. polyp fully open, rich colour and full tissue coverage) colonies of *Duncanopsammia axifuga* were collected within the vicinity of Cairns on the Great Barrier Reef. Colonies were acclimatized to 26 °C for at least two months in a 1200 l outdoor tank at the National Marine Science Centre, Coffs Harbour (NSW, Australia). Flow-through seawater was sourced from Charlesworth Bay (30°16′0.91″S, 153° 8′25.27″E), passed initially through a sand filter, and then further filtered in tanks using a zeolite media filter (JNS model FR-2E, Japan), an activated carbon media filter (JNS model FR-2E, Japan), a cartridge particle filter (EMAUX® model CF25) and an insump protein skimmer (JNS model SK-6, Japan). The protein skimmer and cartridge filter were cleaned once a week. The zeolite media and activated carbon were changed one day before the start of the experiment.

Temperature was maintained at 26 ± 0.5 °C using a heat pump (2100 l/h, EVO-F5, Australia). Ammonia (media±SD) (0.03 ± 0.03 mg/l N), nitrate (0.01 ± 0.02 mg/l N), nitrite (0.002 ± 0.001 mg/l N), phosphorus (0.03 ± 0.01 mg/l PO₄), alkalinity (135 ± 15 mg/l CaCO₃) and calcium levels (405 ± 45 mg/l CaCO₃) were measured every week with a photometer system (Palintest® model 7100, UK). Salinity (35.9 ± 0.57 ppm) and pH (8.27 ± 0.11) were monitored constantly using probes located in the main tank (Apex controller, Neptune Systems, USA). Superficial photosynthetically active radiation (PAR) was measured, at coral fragment level, at 12:00 PM (640.4 ± 311 μmol photons m⁻² s⁻¹) using a light meter (LI-COR model 250A, Canada). Dissolved oxygen (8.76 ± 0.15 mg/l) was measured with a handheld meter (HORIBA model U− 50, Japan). Values of ammonia, nitrate and nitrite are expressed in mg/l N following the recommendations of Chapman (1998). Colonies were fragmented using a diamond wheel (EZ545 Lock™, USA) attached to a cordless Dremel® (model 8220, USA) into 54 similar sized pieces (~1 cm). All fragments were fixed using one drop of cyanoacrylate glue (Gel control, Loctite®) on top of glass tile stoppers (Vinolok, Czech). Trial experiments demonstrated that these tiles do not lose weight over time and are less likely to be overgrown by algae.

Three different types of fragments and fixing position were tested: i) trunks initially without polyp, fixed upside-down; ii) trunks initially without polyp, fixed the right way up; and iii) trunk initially with one polyp (all former type of fragments were fixed the right way up, thus it will not be mentioned it again). All fragments were randomly assigned to the different treatments, and transferred into independent vials of
250 ml, provided with flow-through water (flow rate: ~ 0.6 ml s\(^{-1}\)) and placed in a water bath on top of the 1200 l tank, without an acclimatization period to evaluate post-fragmentation survival, every 20 days up to 100 days. Every vial had an independent air and water hose. Corals were placed at 5 cm water depth.

### 3.2.2 Feeding regimes

The effects of coral feeding regimes were tested using three different diets: i) unfed; ii) normal *Artemia*; and iii) lipid-enriched *Artemia.* *Artemia* was used because it is a commonly used live food in aquaculture facilities (Dhont and Sorgeloos 2002), well accepted by several corals species (Houlbrèque *et al.* 2003; Yii-Siang *et al.* 2009) and specifically by *D. axifuga* as corroborated in Chapter 2. *Artemia* diet can enhance coral growth and survival (Petersen *et al.* 2008). Lipid-enriched *Artemia* was used because it improves overall coral health (Lim *et al.* 2017). Corals in *Artemia* treatment were fed every three days for 1 hour (at 17:00) using two-day old *Artemia,* previously hatched from *A. salina* cysts (‘AAA’ grade GSL *Artemia* Cyst, INVE, Belgium). A magnetized cyst collector tube was used to remove unhatched cysts (Sep-Art\(^{™}\), INVE, Belgium). The lipid-enriched *Artemia* were placed for 5 hours in 10 litres of seawater containing 2 g of a suspension of an enrichment product (Selco\(^{®}\) S.presso, INVE, Belgium) with high levels of essential polyunsaturated fatty acids (C22:6n-3 = 260 mg·g\(^{-1}\); C20:5n-3 = 25 mg·g\(^{-1}\)) that had been previously mixed in a blender with half a litre of freshwater (2 minutes). During the feeding hour, water supply was stopped and then reconnected to eliminate the remaining *Artemia* via overflow. Once a week, vials were replaced by clean ones and randomly moved to avoid any position effect.

### 3.2.3 Laboratory analyses

Buoyant weight (BW) of each coral was measured every 20 days using an analytical balance (0.001 g resolution) with a weighing hook (Ohaus model PA213, USA) (Davies 1989). Tiles were cleaned prior to weighing and water volume and temperature were kept constant throughout the measurements. Growth rate was expressed as mg of mass (skeleton and tissue) incremented per g of initial weight per day (Movilla *et al.* 2012). New polyps were also counted to evaluate polyp production. Additionally, coral colour was measured using the Coral Health Monitoring Chart (Fig. 3.1) (Siebeck *et al.* 2006).

Prior to tissue removal, maximum quantum yield (Fv/Fm) of photosystem II was measured, using an Underwater Fluorometer DivingPAM (©Heinz Walz GmbH,
Germany). All samples were prior dark-adapted for 1 hour. PAM setting was selected based on a previous test (with spare corals of the same species), using three published settings (Ganase et al. 2016; Hill and Scott 2012; Humanes et al. 2016). The final setting was selected to avoid low signal or overflow readings (measuring light intensity 3, damping 2, gain 6, actinic light intensity 12, saturation pulse intensity 8, and saturation pulse width 0.8).

At the end of the experiment (day 100), tissue was removed using a 5 ml pipet tip attached to an air pistol (WJI602-05, Black Ridge, Australia) connected to an air compressor (5.5 HP, Peerless, Australia). The tissue was immediately frozen in liquid nitrogen, and stored at −80 °C. The following week, 1.5 ml of filtered seawater (FSW) was added to each sample and homogenized for 10 seconds using an Ultra-Turrax (TP18-10/S25N-10G, ®IKA, China). The mix was centrifuged for 5 minutes at 1000 rpm and the supernatant was discarded. The pellet (containing symbiont cells) was resuspended in 1.5 ml of FSW and centrifuged again (5 minutes at 1000 rpm). This process was repeated twice and at the end resuspended in 3 ml of FSW. For each sample, a 1 ml subsample was placed in an Eppendorf and stored at 4 °C for symbiont density evaluation during the next two days, using a Neubauer improved cell chamber (Marienfeld, Germany).

The remaining 2 ml of the symbiont tissue samples was centrifuged again for 10 minutes at 3400 rpm, supernatants were discarded and pellets resuspended in 3 ml of acetone at 100 % and stored at 4°C in the dark for 24 hours for chlorophyll content measurements. Samples were centrifuged (10 min at 3400 rpm) and 1.5 ml of each sample was placed in a glass cell (type 1/G/10/CARY) and the optical density was measured in a spectrophotometer (Anadéo Plus 230 V, Bibby Sterilin) at 630 and 663 nm. Chlorophyll a was later quantified following the Jeffrey and Humphrey (1975) equation for dinoflagellates in 100 % acetone (chlorophyll a = 11.43 E_663 - 0.64 E_630; E_x denotes the reading obtained from the spectrophotometer at each specific wavelength).

### 3.2.4 Statistical analyses

Permutational analysis of variance was used to test the effects of type of fragment/fixing orientation (Fixed, 3 levels) and feeding regime (Fixed, 3 Levels) on each univariate response variable (polyp production, growth, algal symbiont density, chlorophyll, maximum quantum yield and colour). In total, the experiment design had nine treatments with six replicate coral fragments in each treatment (n = 6). All analyses were conducted in PRIMER (Anderson et al. 2008) using Euclidean distance similarity matrices and 9999 permutations. When significant differences were found, a post-hoc
pair-wise test was undertaken. All dead fragments were excluded from the final analysis (except in survival).

Homogeneity of variances was assessed prior to analysis with PERMDISP tests. Where these tests were significant, data were transformed to Ln (X + 1) prior to analysis to reduce heteroscedasticity. In the few cases where transformation could not homogenize variances, data were analysed but the significance level was increased to a more conservative \( p < 0.01 \). This approach is appropriate because PERMANOVA is robust to variance heterogeneity with balanced designs (Anderson and Walsh 2013).

**Figure 3.1** Example of fragments from treatments with maximum polyp production observed for *Duncanopsammia axifuga* after 100 days in *ex situ* culture conditions using different types of fixing methods and feeding regimes. From top to bottom: trunk initially without polyp/ upside-down oriented/unfed regime (# new polyps = 6); trunk initially without polyp/right way up oriented/lipid-enriched *Artemia* diet (# new polyps = 7); trunk with one polyp/right way up oriented/normal *Artemia* diet (# new polyps = 5).
3.3 Results

3.3.1 Survival and health indicators

*Duncanopsammia axifuga* showed an overall survival of 85% across all treatments and conditions, with no individual treatment exhibiting survivorship of < 67%. The highest survival rates (100%) were obtained under the following conditions: (1) trunks initially without polyp/upside-down/unfed; (2) trunks initially without polyp/right way up/fed with normal *Artemia*; and (3) corals initially fixed with one polyp, regardless of feeding regime (Fig. 3.2). Coral health indicators obtained using non-invasive (colour chart, fluorescence) and invasive (chlorophyll and algal symbiont density) methods showed relatively similar patterns, validating the use of non-invasive methods for coral aquaculture. Fragment and feeding type produced significant effects on these response variables (Table 3.1). Specifically, fragments initially fixed with one polyp scored significantly higher levels on colour chart, fluorescence, chlorophyll and algal symbiont density. *Artemia* diets (non-enriched and lipid-enriched) also produced significantly greater ranks of algal symbiont density, chlorophyll and final colour. Furthermore, the lipid-enriched diet showed significantly higher maximum quantum yields (Fig. 3.3).

![Figure 3.2](image)

**Figure 3.2** Survival and polyp production (mean ± SE) after 100 days of *ex situ* culture conditions for the scleractinian coral *Duncanopsammia axifuga*, using three different coral fragment types and fixing methods: A) trunk initially without polyp, upside-down oriented, B) trunk initially without polyp, right way up oriented; and C) trunk with one polyp, right way up oriented; as well as three different diets (unfed, normal *Artemia* and lipid-enriched *Artemia*).
### 3.3.2 Polyp production

The production of polyps interacted significantly between the fragment type and feeding conditions (Table 3.1). The quickest polyp production was observed for trunks initially fixed without polyps right way up, showing brand new polyps at 20 days; followed by trunks initially without polyps upside-down oriented, which produced new polyps before 40 days; and lastly corals initially fixed with one polyp, which showed the first new polyps around day 60 (Fig. 3.2). Post-hoc tests, revealed no significant differences throughout the three diet regimes for trunks initially without polyps (right way up or upside-down). It should be noted that all trunks without polyps developed new polyps between 20 and 40 days. However, high variability was observed among replicates, with maximum polyp production recorded for trunks initially without polyp, right way up and fed with enriched *Artemia* (maximum number of polyps [max polyps], mean ± SE) (7, 3.5 ± 1.3), followed by trunks initially without polyp that were upside-down and unfed (6, 3.8 ± 0.7). Post-hoc analyses revealed significantly greater polyp production for trunks with one polyp when fed with enriched *Artemia* (4, 4.0 ± 0.0) and non-enriched *Artemia* (5, 3.0 ± 0.6), compared to the unfed regime, where no new polyps were produced (0, 0 ± 0.0) (Fig. 3.4).

**Table 3.1** Summary of the permutational analysis of variance results for the scleractinian coral *Duncanopsammia axifuga* after 100 days of *ex situ* culture conditions using three different coral fragment types and fixing methods: i) trunk initially without polyp, right way up oriented, ii) trunk initially without polyp, upside-down oriented, and iii) trunk with one polyp, right way up oriented; as well as three different diets (unfed, normal *Artemia* and lipid-enriched *Artemia*).

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<tr>
<td>Maximum quantum yield (Fv / Fm)</td>
<td>6.752</td>
<td><strong>0.0030</strong></td>
<td>11.576</td>
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</tbody>
</table>

Significant differences are indicated by bold
3.3.3 Growth rate

During the experiment, *D. axifuga* increased biomass in all treatments. There were significant interactions in coral growth between fragment type and feeding conditions (Table 3.1). The highest growth rates were observed for trunks with one polyp, fed with enriched *Artemia* (0.0029 ± 0.0001 mg/day), followed by trunks with one polyp, fed with normal *Artemia* (0.0025 ± 0.0005 mg/day). When the coral fragments without polyps were compared, the fastest growth rates were obtained for trunks initially without polyps, right way up oriented and fed with enriched *Artemia* (0.0008 ± 0.0004 mg/day), and for trunks without polyps, upside-down and fed normal *Artemia* (0.0008 ± 0.0005 mg/day). The slowest growth rate was registered for trunks without polyps, right way up and not fed (0.0002 ± 0.0002 mg/day) (Fig. 3.4).

![Figure 3.3](image_url)

**Figure 3.3** Maximum quantum yield (Fv / Fm), algal symbiont density, coral colour health monitoring chart and chlorophyll *a*, after 100 days of *ex situ* culture conditions for the scleractinian coral *Duncanopsammia axifuga*, using three different coral fragment types and fixing methods: i) trunk initially without polyp, upside-down oriented (UD), ii) trunk initially without polyp, right way up oriented (N), and iii) trunk with one polyp, right way up oriented (Polyp); as well as three different diets: unfed (U), normal *Artemia* (NA) and lipid-enriched *Artemia* (EA). Bars represent the means (+ SE) of the main effects, as there were no significant interaction between fixing method and feeding treatment. Significant groups (at *p* < 0.05), derived from pairwise post hoc tests, are indicated with different letters.
Figure 3.4 Growth rate and polyp production after 100 days of *ex situ* culture conditions for the scleractinian coral *Duncanopsammia axifuga*, using three different coral fragment types and fixing methods: i) trunk initially without polyp upside-down oriented (upside down trunk), ii) trunk initially without polyp-right way up oriented (normal trunk); and iii) trunk with one polyp-right way up oriented (Polyp); as well as three different diets (unfed, normal *Artemia* and lipid-enriched *Artemia*). Bars represent the means (+ SE) of the main effects, as there were no significant interactions between fixing method and feeding treatment. Significant groups (at $p < 0.05$), derived from pairwise post-hoc tests, are indicated with different letters.

### 3.4 Discussion

This study represents the first detailed aquaculture experiment of *D. axifuga*. The use of coral fragments without polyps from this species was found to be an appropriate technique to increase polyp production during cultivation. Previously these trunks were deemed a waste product of the fragmentation process in traditional coral aquaculture. At the same time, it was demonstrated that fixing corals upside-down (mimicking processes that could occur after severe weather conditions with fragments getting stuck in reef
crevices) can be beneficial, as corals were able to survive and produce relatively high rates of polyp production.

Coral survivorship following fragmentation is an important factor for coral aquaculture and reef restoration projects. Some studies in the wild report no survivorship in the transplanted colonies after six months (Bak and Criens 1981), while others obtained up to 83 % survival after three years (Guzmán 1991). In situ intensive coral farming has achieved 34 % survival after a year (Bongiorni et al. 2011), whereas ex situ initiatives have shown around 78 % after 10 months of culture (Forsman et al. 2006), between 52 and 83 % after 85 days (Schlacher et al. 2007) and up to 90 % after a week (Shafir et al. 2006a). The present results, after 100 days of culture, demonstrated an overall survivorship of 85 % (including all treatments combinations). Furthermore, all the treatments using fragments initially fixed with one polyp had 100 % survivorship. There may be a number of factors contributing to the high survivorship in this experiment including water quality and relatively long acclimatization time of the mother colonies into the tanks, prior to the fragmentation process.

As with previous work (Petersen et al. 2008), feeding of Artemia enhanced coral growth. For example, the growth rate of corals with trunks that had one polyp was significantly faster for fed than unfed individuals, with the fed ones producing the fastest growth rate among all treatments tested. Feeding D. axifuga with Artemia may, therefore, be used to enhance growth and polyp production in aquaculture. However, the enriched diet did not elevate coral growth when compared with unenriched Artemia. In terms of coral production under ideal conditions, there may be limited advantage of using a lipid-enriched diet, especially given the additional cost. However, as environmental conditions become more stressful (e.g. during transplantation) it is likely that the lipid-enriched Artemia diet will enhance coral resilience and improve coral health (Lim et al. 2017).

The speed at which coral polyps were produced across the trunks with or without polyps reflects the importance of heterotrophy in the well-being of some coral species. Regardless of the fixing orientation (right way up or upside-down), fragments initially without polyps exhibited the highest polyp production recorded (max polyps = 7), even more than those fragments initially fixed with one polyp. These results might seem contradictory, but in trunks initially fixed without polyps, brand new polyps started growing at the base and top of the trunks, doubling polyp production. In contrast, fragments with one polyp only produced new polyps surrounding the already existent polyp at the top of the fragment, but not at the base. These results cannot be compared to other studies, as similar experiments are not in evidence in the literature. Trunk
orientation may, therefore, be a useful technique for commercial coral aquaculture if elevated early polyp production leads to higher growth and survivorship in the long term.

In developing new techniques to optimise polyp production and growth in *D. axifuga*, I assessed the potential of this species for commercial aquaculture. Throughout the experiment, *D. axifuga* demonstrated high survivorship and positive growth across all treatments. Diet regimes showed significant effect on trunks initially fixed with one polyp, but not in trunks without polyps, and as consequence, the feeding selection should be adapted to the developing stage of the corals being cultured. Finally, it is clear that the fragments of *D. axifuga* without polyps should not be considered as waste, as they can quickly produce new polyps and contribute to production.
Chapter 4 - A potential method for improving coral self attachment

This chapter has been modified, to comply with the University thesis guidelines, from the following published paper:


doi:10.1111/rec.12698
ABSTRACT

Coral restoration is becoming increasingly important to sustain declining reefs. The survival rate of translocated corals in restoration projects is around 65%. This rate is, however, highly variable among projects, with success ranging from 0 to 90% and with detachment being a significant cause of mortality. Improving the speed and strength of coral self-attachment would increase survivorship in translocated corals. To address this need, using ex situ laboratory experiments I tested whether fragments of the scleractinian coral, *Hydnophora rigida*, artificially attached upside-down would self-attach more rapidly to the substratum than those artificially attached the right way up, which is the normal practice. I also tested the effect of three different diets (unfed, normal *Artemia*, and lipid-enriched *Artemia*) on coral growth and other biological responses. After 100 days, the results demonstrated that corals fixed upside-down grew significantly wider and faster over the substratum than corals fixed the right way up. A significantly higher number of fragments fixed upside-down were also able to self-attach and grow over the substratum (87%) compared with fragments fixed the right way up (58%). Neither the buoyant weight, height increment, algal symbiont density, chlorophyll, maximum quantum yield nor colour of corals fixed upside-down differed significantly from corals fixed the right way up. The data shows that simply inverting the orientation of coral fragments may substantially accelerate the time for self-attachment, and increase the survival rate of translocated corals in restoration projects.

4.1 Introduction

Coral reefs are under threat worldwide (Hughes *et al.* 2003; Hughes *et al.* 2017), with an increasing number of coral species at risk of extinction (Carpenter *et al.* 2008). Coral cover in the Caribbean has significantly declined (Gardner *et al.* 2003) and up to 100% mortality has been reported for some coral species along the Pacific coast of Costa Rica (Guzmán 1991). On Australia’s Great Barrier Reef, calcification rates have declined since 1990 due to increasing physiological stresss (De'ath *et al.* 2009), and scleractinian coral cover has been reduced by over 70% (Bell *et al.* 2014). Additionally, the most significant bleaching event ever recorded for the area occurred in 2016 (Hughes *et al.* 2017), with recurrent bleaching in 2017. In response to the declines of coral reefs, coral restoration programs are being used to help maintain coral cover at high value sites (Barton *et al.* 2017; Hein *et al.* 2017; Lirman and Schopmeyer 2016).
Recently, there have been increasing efforts to restore reefs using sexually propagated corals (Chamberland et al. 2015; Cruz and Harrison 2017; Edwards et al. 2015; Guest et al. 2010). However, coral reef rehabilitation usually involves transplanting asexually propagated coral fragments that have been grown out in a nursery, similar to silviculture (Epstein et al. 2001; Rinkevich 2008), and generally shows high variability in restoration outcomes (Barton et al. 2017; Benjamin et al. 2017). Transplantation methods must minimize the detachment losses to achieve success (Gomez et al. 2010). Yet, self-attachment of coral fragments artificially attached (with cyanoacrylate glue, epoxy, cement or cable ties) can be a relatively slow process (even for branching species that use asexual fragmentation as a way of propagation), with most losses occurring in the initial months (Bongiorni et al. 2011; Clark and Edwards 1995; Forrester et al. 2011; Williams and Miller 2010). Especially in high energy areas, losses by wave action tend to be high, regardless of the artificial attachment method (Clark and Edwards 1995; Edwards and Clark 1998; Fisk and Edwards 2010). However, once coral fragments are artificially attached and later self-attach, the rate of loss decreases significantly (Guzmán 1991; Clark and Edwards 1995; Williams and Miller 2010; Forrester et al. 2011) because the fragments are less likely to be removed by waves and currents (Lirman 2000). Consequently, coral self-attachment is a bottleneck in successful coral restoration efforts (Ferse 2010; Jaap 2000) and information on self-attachment is considered vital for successful restoration projects (Bowden-Kerby 2001).

Several restoration projects have grown sexually and asexually produced coral fragments at ex situ nurseries, with larger corals eventually being translocated to the reef (Baria et al. 2012; Boch and Morse 2012; Guest et al. 2014; Ng et al. 2015). For those types of restoration attempts, knowledge of optimal feeding diets is relevant, because heterotrophy supplies a considerable amount of the daily energy required by adult corals and recruits (Houlbrèque and Ferrier-Pagès 2009; Toh et al. 2013a), and time until translocation can be shortened by increasing coral growth through heterotrophic feeding (Barton et al. 2017).

Scleractinian corals in the genus Hydnophora are among the top 20 most traded species in the marine aquarium trade and are included in the top 10 species imported by the United States (Barton et al. 2017). The coral Hydnophora rigida is listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The species has been recently used in coral reef restoration attempts, though with variable success (Bongiorni et al. 2011; Cabaitan et al. 2015; Ng et al. 2015; Toh et al. 2017). Given the potential importance of this coral species in the aquarium...
trade and for reef restoration, there is a need to develop techniques to maximise its production and survival.

Fragment orientation can affect growth and production of branches or polyps (Bruckner and Bruckner 2001; Fong and Lirman 1995; Soong and Chen 2003). However, the effect of artificially attaching coral fragments upside-down on self-attachment has not been rigorously tested before. Accordingly, I used a manipulative experiment to test the hypotheses that fragments of *H. rigida* will self-attach faster and grow more extensively over the substratum when fixed upside-down than when fixed the right way up. In the same experiment, I also evaluated the effect of three different feeding regimes on coral fragment self-attachment, as well as a range of morphological and physiological responses.

### 4.2 Material and methods

Six colonies of *H. rigida* were collected from the vicinity of Cairns on the Great Barrier Reef (Queensland) and transported to the National Marine Science Centre, Coffs Harbour (New South Wales). Colonies were acclimatized for at least two months in a 1200 L outdoor tank. Seawater, sourced from Charlesworth Bay (30°16'0.91"S, 153°8'25.27"E), and passed through the same filtration system referred to in Chapter 3. As this experiment was performed simultaneously with the experiment from Chapter 3, the quality water monitoring methods and related results (salinity, pH, temperature and nutrients) were the same.

Colonies were fragmented into 48 similar sized pieces (~3 cm), using a diamond wheel (EZ545 LockTM, USA) attached to a cordless Dremel® (model 8220, USA). Twenty-four tip fragments were fixed the right way up and the remaining 24 were fixed upside-down (after the tip was cut off) using one drop of cyanoacrylate glue (Gel control, Loctite®) placed in the middle of the cut or open skeleton (without tissue at the fixing point for both types of fixing method) and then held steady for 5 seconds on top of glass stoppers (Vinolok, Czech).

The experiment had two orthogonal factors: (a) fixing orientation (2 levels - right way up, and upside-down); and (b) feeding regime (3 levels – unfed, fed normal *Artemia*, and fed lipid-enriched *Artemia*). In total, the experimental design had six treatments with eight replicate coral fragments for each treatment (n = 48). Fragments from different colonies were randomized among treatments using numbers generated in Excel, but ensuring each treatment had at least one fragment from each colony. All fragment
replicates were assigned into independent vials of 250 ml, without acclimatization, to evaluate post-fragmentation survival at day 100. Every vial was provided with air and flow-through seawater. Additionally, three fragments fixed the right way up and three fragments fixed upside-down were kept for 15 months (fed two times a week with non-enriched Artemia) to detect possible coral self-attachment changes over a longer term period (all these fragments were derived from different colonies). The Artemia feeding treatments were prepared following the same procedures detailed in Chapter 3. Corals in the Artemia treatments were fed using a concentration of 40 individual ml⁻¹, every 3 days for 1 hour at 17:00.

Basal growth was defined as the growth in area (mm²) of the base of the fragment, measuring the longest diameter observed over the tile and calculated through the formula (Area = π × radius²). Linear increment in height was measured from the top to the bottom of the fragment (Ferse and Kunzmann 2009; Gomez et al. 2014). All length measurements were done using digital Vernier calipers (to nearest 0.1 mm). Buoyant weight (BW) (Jokiel et al. 1978) was measured using an analytical balance (± 0.001 g) with a weighing hook (Ohaus model PA213, USA). The tiles on which the corals were fixed were cleaned to prevent weight gain by any biofouling addition. Water temperature and volume were kept steady during the BW measurements. Growth (basal growth, height increment and buoyant weight) was calculated by subtracting the measurement at day 1 from that at day 100. To convert to monthly rates, these values were multiplied by 30/100 (assuming an average month to be 30 days long).

Self-attachment was measured every 20 days by checking if the fragments were clearly growing or not growing over the primary substrata at least at one point of the tile. A score was assigned to define coral self-attachment (i.e. 0 = no self-attachment; 1 = coral tissue clearly growing over the primary substrata at least at one point of the tile). Additionally, because the surveys were performed at 20 day intervals, the accurate time of self-attachment could not be determined, therefore successful self-attachment was assumed at the mid-point of time among surveys, following Guest et al. (2011). To detect differences in the rate of self-attachment between upside-down and right way up fragments, Kaplan-Meier survival analysis (log-rank test) was performed using Sigma Plot 13.0 (non-attached fragments were assigned as censored data, and fragments that self-attached were considered as an event of interest per time step). Median time for the 50 % of fragments to self-attach, standard error and confidence intervals (CI) were also obtained through the Kaplan-Meier analysis.
Coral colour was measured using the Coral Health Monitoring Chart (Siebeck et al. 2006). Colour change was obtained using the formula: colour change = coral colour at day 100 - coral colour at day 1. After 100 days of the experiment, maximum quantum yield ($F_V/F_M$) was measured using an Underwater Fluorometer Diving-PAM (©Heinz Walz GmbH, Germany), using the same procedure and settings from Chapter 3. Afterwards, coral tissue was removed and the tissue was frozen in liquid nitrogen and stored at –80 °C. Algal symbiont density and chlorophyll were calculated following the techniques explained in Chapter 3. Chlorophyll $c_2$ was later quantified following the Jeffrey and Humphrey (1975) equation for dinoflagellates in 100 % acetone (chlorophyll $c_2 = 27.09E+63 - 3.63E+63$; $Ex$ denotes the reading obtained from the spectrophotometer at each specific wavelength). The data were normalized against surface area, obtained using the wax dipping method (Veal et al. 2010).

A chi-square ($X^2$) contingency test was used to test whether the proportion of upside-down corals that self-attached to the substrata was greater than the proportion of corals fixed the right way up that self-attached. Two-way permutational analysis of variance was used to test the effects of the fixing orientation and feeding regime on each univariate response variable (basal growth, buoyant weight, height increment, symbiont density, chlorophyll $a$, chlorophyll $c_2$, total chlorophyll ($a + c_2$), maximum quantum yield and colour change). All analyses were conducted in PRIMER7 and PERMANOVA+ using Euclidean distance similarity matrices and 9999 permutations (Anderson et al. 2008; Clarke and Gorley 2015). The significance level to reject the null hypotheses was set at 0.05. When significant differences were found, appropriate post-hoc pair-wise tests were undertaken. One fragment from the unfed upside-down treatments died during the experiment and was excluded from all the subsequent analysis (e.g. Chi-square test and the Kaplan-Meier test for self-attachment).

### 4.3 Results

After 100 days, *H. rigida* showed an overall survival rate of 98 %. The proportion of corals that self-attached when fixed upside-down (20 corals out of 23; 87 %) versus the right way up (14 corals out of 24; 58 %) differed significantly ($X^2 = 4.81$, 1 d.f., $p < 0.05$).

The median time to self-attach for upside-down fragments (71.3 days, SE = 2.4, CI = 66.7 – 75.9 days) was significantly lower than that obtained for right way up fragments (81.0 days, SE = 1.9, CI = 77.3 – 84.7 days) (log-rank test = 14.339, df = 1, p
< 0.001) (Fig. 4.1). The data on self-attachment for each feeding treatment is presented in Table 4.1

Table 4.1 Attachment success (unit: yes or no) at each survey of Hydnophora rigida during 100 days of ex situ culture using two different coral fixing methods (upside-down and right way up) and three different diets (unfed, normal Artemia and lipid-enriched Artemia). *A dead fragment in this treatment it is not included.

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No significant interactions between the fixing method and feeding type were observed for all the variable responses tested ($p > 0.20$ in all cases) (Table 4.2). Orientation affected coral self-attachment, with upside-down oriented fragments growing significantly wider over the tile than fragments that were fixed the right way up ($p - F_{1,45} = 5.42, p = 0.02$) (Figs 4.2 & 4.3, Table 4.2). However, no significant differences were found between fragment orientations for height increment, buoyant growth rate, symbiont density, chlorophyll ($a, c_2$ or total), colour change and maximum quantum yield ($p > 0.09$ in all cases) (Table 4.2; Figs 4.3 & 4.4).
Figure 4.1 Cumulative self-attachment trajectories for *Hynophora rigida* during 100 days of *ex situ* culture (as the exact time of self-attachment is unknown, mid-points between surveys were used as estimates of times to self-attachment).

Figure 4.2 Coral self-attachment for *Hydnophora rigida* after 100 days of *ex situ* culture conditions with differing artificial attachment orientation and diets: (A) upside-down, unfed; (B) upside-down fed with lipid-enriched *Artemia*; (C) right way up, unfed; and (D) right way up fed with lipid-enriched *Artemia*. 
A significant feeding effect was detected for most of the response variables (except for buoyant weight and maximum quantum yield) (Table 4.2). Corals that were fed with *Artemia* (normal or lipid-enriched) significantly increased their basal self-attachment, height increment, colour, symbiont density and chlorophyll *a* and *c* (Figs 4.3 & 4.4).

The advantages of coral fragments being grown upside-down compared to right way up did not change when the corals were grown for 15 months. Upside-down fragments showed a wider basal self-attachment, even completely overgrowing the edges of the substratum. No differences were observed in the colour of fragments whether fixed right way up or upside-down (Fig. 4.5).

**Figure 4.3** Basal growth, height increment, buoyant weight and colour change, after 100 days of *ex situ* culture conditions for the coral *Hydnophora rigida*, using two different fixing methods (right way up oriented and upside-down oriented) and/or three different diets (unfed, normal *Artemia* and lipid-enriched *Artemia*). Bars represent the means (+ SE) of the main effects, as there were no significant interactions between fixing method and feeding treatment. Significant groups (at *P* < 0.05), derived from pairwise post hoc tests, are indicated with different letters.
Table 4.2 Summary of the permutational analyses of variance of the morphological and physiological variables of the coral Hydnophora rigida after 100 days of ex situ culture conditions using two different coral fixing methods (right way up and upside-down) and three different diets (unfed, normal Artemia and lipid-enriched Artemia).

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<td>Height increment</td>
<td>0.138</td>
<td>0.711</td>
<td>3.276</td>
<td>0.049</td>
<td>0.699</td>
<td>0.502</td>
</tr>
<tr>
<td>Buoyant weight</td>
<td>0.001</td>
<td>0.998</td>
<td>1.310</td>
<td>0.289</td>
<td>0.070</td>
<td>0.945</td>
</tr>
<tr>
<td>Algal symbiont density</td>
<td>&lt; 0.001</td>
<td>0.986</td>
<td>3.181</td>
<td>0.042</td>
<td>1.598</td>
<td>0.221</td>
</tr>
<tr>
<td>Colour change</td>
<td>0.331</td>
<td>0.554</td>
<td>11.750</td>
<td>&lt; 0.001</td>
<td>0.086</td>
<td>0.913</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.598</td>
<td>0.443</td>
<td>3.356</td>
<td>0.039</td>
<td>0.333</td>
<td>0.728</td>
</tr>
<tr>
<td>Chlorophyll c₂</td>
<td>3.066</td>
<td>0.091</td>
<td>3.072</td>
<td>0.049</td>
<td>0.829</td>
<td>0.448</td>
</tr>
<tr>
<td>Total chlorophyll (a + c₂)</td>
<td>1.651</td>
<td>0.210</td>
<td>3.452</td>
<td>0.038</td>
<td>0.517</td>
<td>0.604</td>
</tr>
<tr>
<td>Max quantum yield (Fv / Fm)</td>
<td>0.073</td>
<td>0.798</td>
<td>2.653</td>
<td>0.080</td>
<td>0.309</td>
<td>0.734</td>
</tr>
</tbody>
</table>

Figure 4.4 Symbiont density, maximum quantum yield, chlorophyll a and c₂ after 100 days of ex situ culture conditions for the coral Hydnophora rigida, using two different coral artificial attachment orientation methods (upside-down and right way up) and/or three different diets (unfed, normal Artemia and lipid-enriched Artemia). Bars represent the means (+ SE) of the main effects, as there were no significant interactions between fixing method and feeding treatment. Significant groups (at p < 0.05), derived from pairwise post-hoc tests, are indicated with different letters.
4.4 Discussion

Interventions on coral reefs for restoration purposes either involve supplying reefs with competent larvae, or artificially attaching sexually and asexually propagated corals to the reef (Guzmán 1991; Guest et al. 2011; 2014). The success of either asexually or sexually produced corals is dependent on the self-attachment and growth of coral over the substratum (Forrester et al. 2011; Guest et al. 2011). Given that coral fragments fixed upside-down self-attached faster and grew significantly wider at the base than fragments fixed the right way up, this simple method of artificial attachment may have benefits for coral restoration projects. The “upside-down technique” may also help to speed up the time until fragments can be exported from coral production into the ornamental aquaculture industry. However, a survey about the marketability of upside-down fragments in the aquarium trade needs to be considered.

Importantly, the upside-down method did not cause significant differences with respect to other biological factors when compared with corals fixed the right way up. For example, corals fixed upside-down grew to similar height and weight, as well as having similar colour, algal symbiont density, chlorophyll \( a, c_2 \text{ and total} \), survival and maximum quantum yield, when compared with corals fixed the right way up. Similar
results were observed by Bruckner and Bruckner (2001), finding no differences in survivorship or length increases for *Acropora palmata* with different fragment orientation. Other studies have also shown that corals are able to adapt and produce new branches regardless of orientation (Soong and Chen 2003). However, the utility of the upside-down technique still needs to be evaluated for other coral species and tested in field-based coral restoration trials. Because cyanoacrylate glue is relatively ineffective underwater, it would be necessary to use another artificial attachment method (e.g. marine epoxy, cement, cable-ties) for such trials.

Results in terms of survival during reef restoration and *in-situ* mariculture are variable with many factors influencing the outcomes (Cabaitan *et al.* 2015; Young *et al.* 2012). A recent review has calculated a median survival rate for corals during restoration projects of around 65 %, but there is high variability of success (0 to 90 %) among different projects (Bayraktarov *et al.* 2016). Fragments artificially attached to the reef have significantly higher survival than non-attached fragments (Forrester *et al.* 2011), and several studies have highlighted the importance of coral self-attachment for coral reef restoration success (Harriott and Fisk 1987; Kaly 1995; Edwards and Clark 1998; Lindahl 1998; Shafir *et al.* 2010). For *H. rigida*, Bongiorni *et al.* (2011) reported high levels of detachment between two study sites (40 % and 70 % in 6 months) using superglue to artificially attach the coral fragments to plastic pins. However, about 90 % self-attachment success was observed after seven months for corals artificially attached with epoxy on dead giant clam shells in the Philippines (Guest *et al.* 2011). Although comparison among restoration projects are difficult due to differences in environment and methodologies (Clark and Edwards 1995), the variability of self-attachment results reported among projects emphasizes the need for careful attention to artificial attachment techniques.

Guest *et al.* (2011) found a median self-attachment time for *H. rigida* of 117 days for small fragments and 52 days for larger fragments attached with epoxy to dead giant clam shells on the reef. The present study found for similarly-sized small fragments (~ 3 cm) a median time to self-attachment for upside-down fragments of 71 days in *ex-situ* culture. Our survival rate (98 % after 100 days) was similar to that observed for transplanted *H. rigida* in the Philippines (Guest *et al.* 2011). Thus, in projects requiring restoration of *H. rigida*, the species appears amenable to transplantation with regard to both survival and ability to self-attach.

Heterotrophy is important for corals because it provides additional energy for growth, as well as nutrients not supplied by the endosymbiotic algae (Houbrèque and
Survival and growth of propagated corals can be improved through feeding (Barton et al. 2017), by reducing the time spent as fragile early stages (Petersen et al. 2008) and increasing coral health and resilience; both are important considerations during restoration projects and in aquaculture production (Lim et al. 2017). In this study, there were clear benefits of feeding on the growth and colour of corals, suggesting that this may be a useful way to improve resilience prior to transplantation. However, no differences in growth were detected between corals fed with normal Artemia and lipid-enriched Artemia. This contrasts with previous studies that demonstrated how corals fed with a lipid-enriched diet have higher energy reserves and improved resilience, especially when this diet is supplied before and during stress episodes (Lim et al. 2017; Tagliafico et al. 2017).

Given the increasing stress predicted for coral reefs in coming decades (Hughes et al. 2003; Mumby 2017), management interventions are necessary to conserve these ecosystems. Although not practical on large geographic scales, active coral restoration activities can be used to conserve high value coral reef sites (Lirman and Schopmeyer 2016). The upside-down method increased coral self-attachment rates and enhanced basal growth. It may, therefore, be a simple but useful method to improve success in coral reef restoration and in situ nursery efforts using coral fragmentation.
Chapter 5 - Lipid-enriched diets reduce the impacts of thermal stress in corals

This chapter has been modified, to comply with the University thesis guidelines, from the following published paper:


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ABSTRACT

Coral species are better able to survive bleaching events with heterotrophic feeding, which raises the level of lipid provisions and coral resilience against stress episodes. Here I report that a lipid-enriched diet allowed two coral species (*Duncanopsammia axifuga* and *Acropora millepora*) to resist an experimental bleaching event that involved exposure to temperatures of 32 °C for 14 days. The diet, containing *Artemia* enriched with omega-3 polyunsaturated fatty acids (PUFAs), increased the proportions of essential fatty acid within coral tissue at normal temperatures, and maintained normal levels of these fatty acids under high temperatures. Neither of the two species significantly bleached when fed an enriched diet, and *D. axifuga* also grew faster, and increased colour, chlorophyll, algal symbiont density and PUFA proportions in the enriched diet compared to controls. Overall, this study sheds new light on the role of heterotrophic feeding in coral resilience to bleaching and provides a novel approach for bleaching prevention, reef restoration and improved coral aquaculture.

5.1 Introduction

Although it is well established that corals are vulnerable to rising sea temperatures (Hughes *et al.* 2003; Hughes *et al.* 2017; Palumbi *et al.* 2014), ongoing research is revealing various adaptations that allow corals to cope with temperature stress (Boulotte *et al.* 2016; Hoey *et al.* 2016; Logan *et al.* 2014). However, the rate of ocean warming may be progressing faster than corals can evolve adaptive responses (Eakin 2014; Hughes *et al.* 2017), with an increased frequency of bleaching events and reduced time for coral recovery (Hughes *et al.* 2017). Human intervention via reef restoration may be an important measure for allowing coral ecosystems to survive (Rinkevich 2014). Here, I explore the potential of artificial heterotrophic feeding in facilitating resistance to thermal stress in corals as an adjunct to reef restoration endeavours.

Corals depend on energy reserves, and particularly on their nutritional lipid stores, to survive bleaching events (Baumann *et al.* 2014; Grottoli and Rodrigues 2011; Rodrigues *et al.* 2008). This is because stress induces metabolic alterations that activate mechanisms to catabolize lipid reserves (Hillyer *et al.* 2017). Species that can utilize or increase heterotrophic feeding in the absence of symbiont-derived nutrition are better adapted to survive and will recover faster after a bleaching event (Grottoli *et al.* 2014; Rodrigues and Grottoli 2007; Towle *et al.* 2015), by consuming a diverse array of organisms from bacteria to mesozooplankton, as well as dissolved and particulate organic...
matter (Anthony 1999; Houlbrèque and Ferrier-Pagès 2009; Levas et al. 2016). Even corals receiving abundant light still obtain additional essential nutrients through plankton consumption (Iluz and Dubinsky 2015). By contrast, corals subject to food limitation show increased symbiont degradation due to autophagy (Bodemann et al. 2011), as well as disruption in division cycle synchrony between the host and symbiont due to host-regulated controls in the supply of nutrients to the symbiont (McAuley 1985).

There are clearly positive effects from increased heterotrophy during bleaching events (Grottoli and Rodrigues 2011; Rodrigues et al. 2008), because it allows the coral host to maintain carbon supplies during stressful environmental conditions (Forsman et al. 2012). Bleached corals can rely on lipids obtained through heterotrophy for at least one year (Baumann et al. 2014) and help to maintain the symbiont thylakoid membrane integrity (Tchernov et al. 2004). For example, up to 100% of the nutrients acquired by bleached corals come from heterotrophy, compared to 15 to 35% in healthy corals (Houlbrèque and Ferrier-Pagès 2009). Furthermore, those species adapted to raise their energy reserves by increased heterotrophy under stressful conditions should have long-standing ecological advantages (e.g. more energy to support higher metabolic rates under stressful conditions, and faster recovery after bleaching) (Grottoli et al. 2006).

Bleaching can reduce the level of lipids and the probability of coral survival (Imbs and Yakovleva 2012; Tolosa et al. 2011), with polyunsaturated fatty acids (PUFAs) being the most oxidatively unstable under elevated temperature conditions (Porter 2013). Moreover, coral thermal sensitivity is correlated with the degree of saturation of lipids from the symbiont thylakoid membrane (Tchernov et al. 2004), and Lim et al. (2017) recently demonstrated that supplementary feeding with lipid-enriched Artemia can increase the levels of PUFAs in corals. For this reason, I tested the hypothesis that two coral species (Duncanopsammia axifuga and Acropora millepora) would survive a high temperature episode (two weeks at 32 °C) and/or improve their overall health condition (at 26 °C), when continuously fed with a diet based on Artemia salina enriched with marine-derived lipids, with a high proportion of PUFAs (particularly omega-3 fatty acids, docosahexaenoic [DHA] and eicosapentaenoic [EPA]), relative to a control diet of unenriched A. salina and corals that were not fed.

5.2 Material and methods

5.2.1 Coral fragments and experimental setup

Eight healthy colonies of Duncanopsammia axifuga (large polyp size ~ 10 mm) and six of Acropora millepora (small polyp size ~ 2 mm) were collected from the Great
Barrier Reef, adjacent to Cairns (Queensland, Australia). Each colony was packaged in a plastic bag full of seawater, covered inside an insulated cooler and transported by air overnight to the National Marine Science Centre (NMSC) (Coffs Harbour, northern NSW, Australia). Once they arrived, coral colonies were acclimated for at least 1 month in 1200 L outdoor tanks with flow-through filtered seawater maintained at 26 °C (to match the ambient temperature at time/point of collection) using a heat pump (2100 l h\(^{-1}\); EVOF5). Every mother colony was fragmented into similar nubbin pieces (≤ 5 cm), using a cordless Dremel® rotary tool (model 8220), and fixed to glass tile mounts using cyanoacrylate glue (Gel control, Loctite®). Nubbins were acclimated in the same tank for at least one further month until all injured tissue was completely regenerated. A total of 72 healthy fragments (36 per species) were randomly selected for the experiment. All corals were ranked medium-high in colour (4 to 5) at the start of the experiment, as compared to the Coral Health Monitoring Chart (Siebeck et al. 2006).

Nubbins were transferred into independent vials of 250 ml, each one with air supply (from an air compressor), and independent irrigation dripper valves (~ 0.6 ± 1.2 ml s\(^{-1}\)) delivered flow-through seawater from Charlesworth Bay, Solitary Islands Marine Park, adjacent to the NMSC (30° 16’ 0.91’’ S, 153° 8’ 25.27’’ E). Water was filtered through a gravel filter, sand filter and 10 µm cartridge filter. Photo period was set at 12 h d\(^{-1}\) by 4 overhead metal halide lamps at an average of 280 ± 50 µmol m\(^{-2}\) s\(^{-1}\).

Using 12 replicates per species for each of the 3 different treatments: (1) unfed, (2) two day old normal (unenriched) *Artemia* and (3) lipid-enriched two day old *Artemia*; corals were initially kept for 10 days under ambient temperature conditions (26 ± 0.5 °C) and fed every day at 17:00 h for 1 hour using an optimum feeding rate of 40 ind. ml\(^{-1}\) for *A. millepora* and 90 ind. ml\(^{-1}\) for *D. axifuga*. After the 10 day period, water temperature for half of the corals within each feeding treatment (n = 6 replicates per species) was elevated to 32 °C (1 °C rise per day), using heater-chiller units (Aquahort) to manipulate and maintain both ambient and elevated temperatures. Corals were maintained under the same daily feeding regime, and the experiment was stopped after 14 days.

Coral growth rate was calculated by measuring buoyant weight (Jokiel et al. 1978) using an analytical balance (0.001 g resolution) with a weighing hook (Ohaus; model PA213), at the beginning and at the end of the experiment. Water volume and temperature were kept constant throughout the buoyant weight measurements, and the tiles on which the corals were attached were cleaned to prevent any bio fouling adding to weight gain. Growth rate was expressed in mg of mass (skeleton and tissue) increment per gram of initial weight per day (Movilla et al. 2012). Additionally, colour was measured at Day 1.
and Day 14 of the experiment, using the Coral Health Monitoring Chart (Siebeck et al. 2006). Coral tissue was removed with compressed air, immediately frozen in liquid nitrogen and stored at −80 °C until laboratory analysis.

5.2.1.1 Lipid enrichment of Artemia

Beginning two days prior to the start of the experiment and then every day, 10 g of A. salina cysts (‘AAA’ grade GSL Artemia cysts; INVE Belgium) were incubated in a 10 L cone tank with high aeration and light, and harvested after 20 hours at 28 °C. A magnetised cyst collector tube (Sep-ArtTM; INVE Belgium) was used during the harvesting, to avoid collecting any unhatched cysts or shells. Nauplii were maintained at a density of 400 to 600 ind ml⁻¹. After 24 hours, water was exchanged and half of the nauplii were enriched using two g of a soluble commercial product (Selco® S.presso, INVE Belgium) containing high levels of essential PUFAs (C22:6n-3 = 260 mg g⁻¹; C20:5n-3 = 25 mg g⁻¹), previously mixed for two minutes in a blender with 0.5 l of freshwater. A two-stage enrichment process was used whereby the two day old Artemia nauplii were allowed to feed in eight L of filtered and aerated seawater containing the enriched emulsion for four hours, then the seawater was renewed and another two grams of the mixed enriched emulsion was added and left for another four hours.

In order to confirm that the diet was effectively enriched with lipids, prior to starting experiments ~ 180 mg of non-enriched Artemia versus lipid enriched Artemia (n = 3) were extracted using analytical grade chloroform:methanol (1 : 1; Sigma), then derivatised into fatty acid methyl esters (FAMEs) using 2 M sodium hydroxide in methanol followed by 14 % boron trifluoride in methanol (Sigma-Aldrich) and re-extracted in analytical grade hexane by phase partition with saturated sodium chloride solution according to Valles-Regino et al. (2015). The extracts were analysed by gas chromatography/mass spectrometry (GC/MS; Agilent 6890, coupled with an Agilent 5973 mass selective detector). The mass spectra were recorded at 70 eV ionization voltage over the mass range of 35 to 550 Da. Identification of peaks was based on comparison to a marine lipid mixture (PUFA No.1 Marine Source, Analytical Standards, SigmaAldrich) and matched to a mass spectral library (WILEY 275 and NIST98). A comparison of the lipid profile of the enriched and non-enriched Artemia diets is provided in Fig. 5.1, with mean percent composition based on the integrated area under the curve in Appendix 2.
5.2.1.2 Laboratory analyses

Chlorophyll $a$ and $c_2$ were calculated using the Jeffrey and Humphrey (1975) equation, while host soluble proteins were obtained through the Bradford (1976) method and algal symbiont density following the protocol provided by Hill and Scott (2012) ($n = 72$). Lipids from the coral host and algal symbions ($n = 48$) were extracted together based on a modified version (Valles-Regino et al. 2015) of the Folch method (Folch et al. 1957). Lipid yield and protein values were standardized by the weight of coral tissue that was extracted ($\text{mg g}^{-1}$). Chlorophyll and algal symbiont density were standardized taking into account the tissue yield and coral surface area (Veal et al. 2010).

Coral tissue lipid extracts were derivatized into FAMEs and analysed by flame ionisation detection coupled to a gas chromatographer (Agilent 6890N) (Valles-Regino et al. 2015). Standard FAMEs (SUP ELCO 37-Component FAME Mix CRM47885) and marine lipid mixtures (PUFA No.1 Marine Source, Analytical Standards, Sigma-Aldrich) were used to calibrate retention times.

Figure 5.1 Coral feeding diets: non-enriched Artemia vs. lipid-enriched Artemia fatty acid profiles and a commercial enrichment diet profile (major fatty acids peaks have been labeled).
5.2.1.3 Statistical analyses

Permutational multivariate analysis of variance (PERMANOVA) was used to test hypotheses about the effects of feeding regimes and temperatures on each univariate response variable (chlorophyll, algal symbiont density, proteins, growth, final colour, each type of identified fatty acid (FA), and the percent composition of total FAs). Multivariate PERMANOVAs were used to test hypotheses about differences in major FA composition among treatments. All PERMANOVA analyses used similarity matrices based on Euclidean distances and 9999 permutations (Anderson et al. 2008). When significant differences were found in PERMANOVA analyses, post hoc pair-wise tests were undertaken when appropriate.

5.3 Results

Corals fed with the lipid-enriched diet had significantly higher colour, and for both species, most (except one Acropora millepora replicate) did not bleach after 14 days at extreme elevated temperatures (Fig. 5.2). By contrast, corals in the unfed treatment at 26 °C remained in the same colour range or one step lower, but were bleached at 32 °C.

The enriched diet for Duncanopsammia axifuga resulted in higher growth, chlorophyll and algal symbiont density compared to unfed coral and corals fed with the normal or unenriched Artemia. However, elevated temperature caused a significant reduction in growth, algal symbiont density and total chlorophyll (Table 5.1, Fig. 5.3). There was no significant effect of temperature or feeding on protein and the total lipid yield extracted from the coral tissue of each species (Tables 5.1 & 5.2, Figs. 5.3 & 5.4). However, diet resulted in a significant change in the relative proportion of major FA classes, with an alteration in the percent composition of saturated fatty acids (SFAs) and unsaturated acids of D. axifuga (Table 5.1, Fig. 5.4).

When fed the enriched diet, D. axifuga had a significantly lower proportion of SFAs and significantly higher PUFAs relative to corals that were unfed or fed with unenriched Artemia (Fig. 5.4, Table 5.3). Omega-3 and -6 FAs were significantly higher in both fed treatments compared to unfed controls (Fig. 5.4, Table 5.3). Temperature had no significant effect on the SFAs or PUFAs in D. axifuga, but caused a slight significant drop in monounsaturated fatty acids (MUFAs) (Fig. 5.4, Table 5.3). For A. millepora, MUFAs were significantly higher in unfed compared to the normal (unenriched) Artemia diet (Fig. 5.4, Table 5.3). In comparison, diet had no significant effect on the relative proportions of SFAs or PUFAs in A. millepora (Fig. 5.4, Table 5.3). However, in this coral species temperature resulted in a significant increase in SFAs and a decrease in PUFAs and omega-3 (Table 5.3, Fig. 5.4). Elevated temperature also significantly
reduced the total chlorophyll and algal symbiont density in *A. millepora*, but these parameters were not affected by diet. Similarly, growth was not significantly affected by diet or temperature in *A. millepora* (Table 5.3, Fig. 5.4).

Multivariate analysis demonstrated that temperature had a significant effect on the major FA composition for both species (Tables 5.1 and 5.2). Additionally, of the 21 specific FAs assessed for *D. axifuga*, three exhibited significant feeding × temperature interactions (C16:0, C18:4n-3 and C18:2n-6). Post-hoc tests investigating these significant interaction terms did not provide clear patterns and were associated with inconsistent trends among feeding treatments at different temperatures (Table 5.4). Another ten FAs showed a significant effect with feeding (e.g. C20: 5n-3 and C22:4n-6 were significantly higher in the enriched diet treatment compare to the other treatments). Additionally, four FAs in *D. axifuga* (C16:1n-7, C20:1n-9, C20:5n-3 and C20:2) significantly decreased with temperature (Table 5.3). For *A. millepora*, four FAs differed significantly among feeding treatments, with C18:1n-9 and C18:0 having the highest values in the unfed treatment; whereas C22:6n-3 and C20:3n-6 showed significantly higher values in the lipid-enriched *Artemia*. Furthermore, seven FAs showed significant differences with temperature, including C14:0 and C16:0 increasing; and C20:1n-9, C20:5n-3, C22:5n-3, C22:6n-3 and C20:3n-6 decreasing at higher temperatures, respectively (Table 5.3). Complete FA profiles across treatments for both species are provided in Appendices 3 and 4.
### Table 5.1 Summary of statistical outcomes for *Duncanopsammia axifuga*

<table>
<thead>
<tr>
<th></th>
<th>Feeding (FED)</th>
<th>Temperature (TEMP)</th>
<th>FED x TEMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pseudo F</td>
<td>p Value</td>
<td>Pseudo F</td>
</tr>
<tr>
<td><strong>UNIVARIATE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>4.8469</td>
<td><strong>0.0170</strong></td>
<td>18.9940</td>
</tr>
<tr>
<td>Protein</td>
<td>0.0114</td>
<td>0.9897</td>
<td>1.3983</td>
</tr>
<tr>
<td>Total Chlorophyll ($a + c_{2}$)</td>
<td>6.1943</td>
<td><strong>0.0067</strong></td>
<td>12.6830</td>
</tr>
<tr>
<td>Algal symbiont density</td>
<td>3.5202</td>
<td><strong>0.0420</strong></td>
<td>55.7110</td>
</tr>
<tr>
<td>Final Colour</td>
<td>28.9080</td>
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<td>12.1430</td>
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<tr>
<td>Total lipid yield</td>
<td>0.1294</td>
<td>0.8778</td>
<td>0.0176</td>
</tr>
<tr>
<td>PUFA</td>
<td>18.1040</td>
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<tr>
<td>Omega-6</td>
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<td>0.2059</td>
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<td>2.3284</td>
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<tr>
<td><strong>MUFAP</strong></td>
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<td>C20:2n-6</td>
<td>29.641</td>
<td><strong>0.0001</strong></td>
<td>0.34708</td>
</tr>
<tr>
<td>C20:2</td>
<td>5.4979</td>
<td><strong>0.0138</strong></td>
<td>5.3376</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major fatty acids (n = 21)</td>
<td>6.9203</td>
<td><strong>0.0001</strong></td>
<td>2.9363</td>
</tr>
</tbody>
</table>

Improving coral aquaculture for reef conservation and the aquarium trade
Figure 5.2 Lipid-enriched diet enhances coral resilience against bleaching: images are representative overall final coral colour (higher number = darker colour) for *Duncanopsammia axifuga* and *Acropora millepora* after 14 d under two different temperatures (26 and 32 °C). Coral pictures were selected from the 32 °C treatment. Bars: means + SE
Figure 5.3 The influence of a lipid-enriched diet on the responses of *Duncanopsammia axifuga* and *Acropora millepora* to extreme temperatures. Growth, total chlorophyll \((a + C_2)\), symbiont density and protein content are shown after 14 days under two different temperatures \((26 \, ^\circ C = black \, bars \, and \, 32 \, ^\circ C = grey \, bars)\) and three different treatments: Unfed, Normal *Artemia* and Enriched *Artemia*. Bars represent the means (+ SE) of the main effects.
### Table 5.2 Summary of statistical outcomes for *Acropora millepora*

<table>
<thead>
<tr>
<th></th>
<th>Feeding (FED)</th>
<th>Temperature (TEMP)</th>
<th>FED x TEMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pseudo F</td>
<td>p Value</td>
<td>Pseudo F</td>
</tr>
<tr>
<td><strong>UNIVARIATE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>1.4261</td>
<td>0.2657</td>
<td>0.9043</td>
</tr>
<tr>
<td>Protein</td>
<td>1.9041</td>
<td>0.1681</td>
<td>0.7068</td>
</tr>
<tr>
<td>Total Chlorophyll ($a + c_2$)</td>
<td>0.9539</td>
<td>0.4039</td>
<td>6.7863</td>
</tr>
<tr>
<td>Algal symbiont density</td>
<td>2.0398</td>
<td>0.1465</td>
<td>9.7516</td>
</tr>
<tr>
<td>Final Colour</td>
<td>7.8200</td>
<td><strong>0.0030</strong></td>
<td>97.885</td>
</tr>
<tr>
<td>Total lipid yield</td>
<td>0.6984</td>
<td>0.5092</td>
<td>0.0105</td>
</tr>
<tr>
<td>PUFA</td>
<td>2.0674</td>
<td>0.1551</td>
<td>8.8414</td>
</tr>
<tr>
<td>Omega-6</td>
<td>1.2405</td>
<td>0.3166</td>
<td>0.2068</td>
</tr>
<tr>
<td>Omega-3</td>
<td>2.5335</td>
<td>0.1089</td>
<td>18.7520</td>
</tr>
<tr>
<td>SFA</td>
<td>1.2504</td>
<td>0.3145</td>
<td>18.8340</td>
</tr>
<tr>
<td>MUFA</td>
<td>4.2778</td>
<td><strong>0.0170</strong></td>
<td>0.2068</td>
</tr>
<tr>
<td><strong>C14:0</strong></td>
<td>1.8215</td>
<td>0.1917</td>
<td>92.3920</td>
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<tr>
<td><strong>C16:0</strong></td>
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<tr>
<td><strong>C18:0</strong></td>
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</tr>
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<td><strong>C20:0</strong></td>
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</tr>
<tr>
<td><strong>C14:1</strong></td>
<td>1.6461</td>
<td>0.2240</td>
<td>0.0647</td>
</tr>
<tr>
<td><strong>C16:1n-7</strong></td>
<td>0.5639</td>
<td>0.5812</td>
<td>0.2605</td>
</tr>
<tr>
<td><strong>C18:1n-9</strong></td>
<td>4.6350</td>
<td><strong>0.0202</strong></td>
<td>0.3999</td>
</tr>
<tr>
<td><strong>C18:1n-7</strong></td>
<td>2.5962</td>
<td>0.1063</td>
<td>1.7918</td>
</tr>
<tr>
<td><strong>C20:1n-9</strong></td>
<td>1.5158</td>
<td>0.2412</td>
<td>5.4704</td>
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<tr>
<td><strong>C18:4n-3</strong></td>
<td>2.2122</td>
<td>0.1287</td>
<td>1.8363</td>
</tr>
<tr>
<td><strong>C20:5n-3</strong></td>
<td>1.3132</td>
<td>0.2949</td>
<td>7.2495</td>
</tr>
<tr>
<td><strong>C22:5n-3</strong></td>
<td>0.7537</td>
<td>0.5022</td>
<td>6.9951</td>
</tr>
<tr>
<td><strong>C22:6n-3</strong></td>
<td>4.2036</td>
<td><strong>0.0371</strong></td>
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<tr>
<td><strong>C18:2n-6</strong></td>
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<td>0.7346</td>
<td>3.6154</td>
</tr>
<tr>
<td><strong>C18:3n-6</strong></td>
<td>0.5551</td>
<td>0.5871</td>
<td>0.6483</td>
</tr>
<tr>
<td><strong>C20:3n-6</strong></td>
<td>4.1669</td>
<td><strong>0.0331</strong></td>
<td>27.988</td>
</tr>
<tr>
<td><strong>C20:4n-6</strong></td>
<td>0.2619</td>
<td>0.7823</td>
<td>1.0407</td>
</tr>
<tr>
<td><strong>C22:4n-6</strong></td>
<td>0.2310</td>
<td>0.8005</td>
<td>0.1955</td>
</tr>
<tr>
<td><strong>C20:2n-6</strong></td>
<td>0.4493</td>
<td>0.6464</td>
<td>0.1424</td>
</tr>
<tr>
<td><strong>C20:2</strong></td>
<td>0.5730</td>
<td>0.5762</td>
<td>0.1835</td>
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<tr>
<td><strong>MULTIVARIATE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major fatty acids ($n = 20$)</td>
<td>1.3843</td>
<td>0.2417</td>
<td>5.6840</td>
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</table>
Figure 5.4 The influence of a lipid-enriched diet on the lipid composition of *Duncanopsammia axifuga* and *Acropora millepora* to extreme temperatures. The proportions of polyunsaturated fatty acids (PUFA), Omega-3 fatty acid, Omega-6 fatty acid, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and lipid yield are shown after 14 days under two different temperatures (26 °C = black bars and 32 °C grey bars) and three different treatments: Unfed, Normal *Artemia* and Enriched *Artemia*. Bars represent the means (+ SE).
Table 5.3 Post-hoc pair-wise test analysis, for the feeding and temperature main effects in *Duncanopsammia axifuga* and *Acropora millepora*. < mean significantly smaller at $p < 0.05$; > mean significantly greater at $p < 0.05$; = no significant differences detected.

<table>
<thead>
<tr>
<th></th>
<th>Unfed vs Normal</th>
<th>Unfed vs Enriched</th>
<th>Normal vs Enriched</th>
<th>26 °C vs 32 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duncanopsammia axifuga</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUFA</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N &lt; E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>OMEGA 6</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>OMEGA 3</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>SFA</td>
<td>U &gt; N</td>
<td>U &gt; E</td>
<td>N &gt; E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>MUFA</td>
<td>U &gt; N</td>
<td>U &gt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Growth</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Total Chlorophyll ($a + c_2$)</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N &lt; E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Algal symbiont density</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
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<tr>
<td>Colour</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
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<td>U &gt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
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<td>Arachidic (C20:0)</td>
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<td>U &gt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>Palmitoleic (C16:1n-7)</td>
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<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Oleic (C18:1n-9)</td>
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<td>U &gt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>cis-Vaccenic (C18:1n-7)</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N &lt; E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>Eicosenoic (C20:1n-9)</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>α-Linolenic (C18:3n-3)</td>
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<td>U &lt; E</td>
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<tr>
<td>EPA (C20:5n-3)</td>
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<td>N &lt; E</td>
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<tr>
<td>G-linoleic (C18:3n-6)</td>
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<td>U &lt; E</td>
<td>N = E</td>
<td>26 = 32</td>
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<tr>
<td>Docosatetraenoic (C22:4n-6)</td>
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<td>U &lt; E</td>
<td>N &lt; E</td>
<td>26 = 32</td>
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<tr>
<td>Eicosadienoic (C20:2)</td>
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<td>U &gt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td><strong>Acropora millepora</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chlorophyll ($a + c_2$)</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Algal symbiont density</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Colour</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>PUFA</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>OMEGA 3</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>SFA</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>MUFA</td>
<td>U &gt; N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Myristic (C14:0)</td>
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<td>U = E</td>
<td>N = E</td>
<td>26 &lt; 32</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
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<td>U = E</td>
<td>N = E</td>
<td>26 &lt; 32</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>U = N</td>
<td>U &gt; E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>Oleic (C18:1n-9)</td>
<td>U &gt; N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 = 32</td>
</tr>
<tr>
<td>Eicosenoic (C20:1n-9)</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>EPA (C20:5n-3)</td>
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<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>DPA (C22:5n-3)</td>
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<td>U = E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>DHA (C22:6n-3)</td>
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<td>26 &gt; 32</td>
</tr>
<tr>
<td>D-g-Linoleic (C20:3n-6)</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N = E</td>
<td>26 &gt; 32</td>
</tr>
</tbody>
</table>
Table 5.4 Post-hoc pair-wise test of the *Duncanopsammia axifuga* fatty acids focusing on feeding (top) and temperature (bottom) following significant Feeding x Temperature interactions. < mean significantly $p < 0.05$; > mean significantly greater at $p < 0.05$; = no significant differences detected.

<table>
<thead>
<tr>
<th>Factor: Feeding</th>
<th>Unfed vs Normal</th>
<th>Unfed vs Enriched</th>
<th>Normal vs Enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (C16:0) at 26 °C</td>
<td>U = N</td>
<td>U &gt; E</td>
<td>N &gt; E</td>
</tr>
<tr>
<td>Palmitic (C16:0) 32 °C</td>
<td>U = N</td>
<td>U &gt; E</td>
<td>N &gt; E</td>
</tr>
<tr>
<td>Stearidonic (C18:4n-3) at 26 °C</td>
<td>U &lt; N</td>
<td>U &lt; E</td>
<td>N = E</td>
</tr>
<tr>
<td>Stearidonic (C18:4n-3) at 32 °C</td>
<td>U = N</td>
<td>U &lt; E</td>
<td>N = E</td>
</tr>
<tr>
<td>Linoleic (C18:2n-6) at 26 °C</td>
<td>U = N</td>
<td>U = E</td>
<td>N = E</td>
</tr>
<tr>
<td>Linoleic (C18:2n-6) at 32 °C</td>
<td>U &lt; N</td>
<td>U = E</td>
<td>N = E</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor: Temperature</th>
<th>Unfed 26 °C vs 32 °C</th>
<th>Normal 26 °C vs 32 °C</th>
<th>Enriched 26 °C vs 32 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (C16:0)</td>
<td>26 = 32</td>
<td>26 &lt; 32</td>
<td>26 &lt; 32</td>
</tr>
<tr>
<td>Stearidonic (C18:4n-3)</td>
<td>26 = 32</td>
<td>26 = 32</td>
<td>26 &gt; 32</td>
</tr>
<tr>
<td>Linoleic (C18:2n-6) at 26 °C</td>
<td>26 = 32</td>
<td>26 &lt; 32</td>
<td>26 = 32</td>
</tr>
</tbody>
</table>

5.4 Discussion

Thermal stress in corals results in bleaching due to the loss of symbiotic algae. It can also simultaneously impact the lipid composition and particularly the PUFAs, which are susceptible to peroxidation (Porter 2013). The present study confirms that corals can assimilate high levels of essential PUFAs from lipid-enriched diets, and this can help compensate against the effects of thermal stress, providing a buffer for maintaining a relatively high proportion of PUFAs in their lipid composition when exposed to elevated temperatures. Corals consuming the lipid-enriched diet had improved colour, which for *D. axifuga* is supported by higher chlorophyll levels and greater algal symbiont densities. This positive effect of heterotrophy on coral symbiont autotrophy is consistent with a recent study by Lim *et al.* (2017), which found elevated photosynthetic rates due to higher symbiont densities in *Galaxea fascicularis* fed with a PUFA-enriched diet. In the present study, neither diet nor temperature had significant impacts on protein and total lipids, but they did alter the FA composition, with temperature significantly decreasing and diet significantly increasing the levels of certain essential FAs (e.g. C22:6n-3 in *A. millepora* and C20:5n-3 in *D. axifuga*). Although there were few significant interactions between diet and temperature, the independent but opposite effects of these factors on a number of parameters clearly points to an overall improved condition of corals fed with a lipid-enriched diet, irrespective of temperature stress. The higher growth rates observed in both
coral species suggests enriched feeding might represent a useful technique for coral aquaculture.

Bleached corals in natural ecosystems consistently show higher amounts of SFAs and MUFAs, as well as a lower amount of PUFAs (Bachok et al. 2006). In the present study, a similar outcome was observed for *A. millepora*, which showed poorer colour, reduced chlorophyll and lower algal symbiont density at higher temperatures, coincident with significantly higher SFAs and lower PUFAs in the FA composition. Bleaching reduces FA content, particularly n-3 PUFA concentrations, but this effect can be alleviated in fed corals, or exacerbated in starved corals under thermal stress (Tolosa et al. 2011). As shown in the experiments on *D. axifuga*, the acquisition of essential FAs through diet might mitigate the temperature effect on the relative proportions of SFAs and PUFAs in the holobiont. This may help shield the coral from total bleaching and keep the symbiosis stable, as supported by increased symbiont densities in *D. axifuga* fed with the enriched diet relative to unfed coral, irrespective of temperature stress. By comparison, the slower feeding species, *A. millepora*, showed no significant effects of feeding on the ratio of SFAs: PUFAs, along with no changes in chlorophyll or algal symbiont. Lim et al. (2017) suggested that some species might require a period of adjustment to assimilate PUFA diet supplements. Nevertheless, the different outcomes for the two species in the study highlights the potential for species-specific positive feedback between heterotrophy, endogenous PUFAs and autotrophy; an effect that has been recently corroborated in another coral species with high ingestion rates when fed PUFA-enriched Artemia (Lim et al. 2017).

The variation in the FA proportions from ‘optimum conditions’ (enriched diet at 26 °C) to ‘extreme conditions’ (unfed at 32 °C) can be attributed to some of their properties and functional roles. FAs are important structural components of cell membranes and the viscosity of the lipid bilayer is influenced by the melting temperature of FAs, which decreases with the number of double bonds (Nelson et al. 2008). Under increasing temperatures, the relative proportion of SFAs in cell membranes increases as a natural adaptive response to thermal stress, to regulate cell membrane permeability and preserve correct physiological functions, i.e. homeoviscous adaptation (Hazel 1995). In addition, FAs are stored as triglycerides which can be metabolised as a source of energy (Nelson et al. 2008). Metabolic rates are higher at elevated temperatures and PUFAs stored as triglycerides can become susceptible to oxidation (Porter 2013) and depletion via gluconeogenesis (Nelson et al. 2008). This may explain why higher SFAs and lower PUFAs were found in *A. millepora* under temperature stress. However for *D. axifuga*,
heterotrophic feeding on the PUFA-enriched diet appears to have helped maintain high PUFA levels, even after two weeks of exposure to elevated water temperatures capable of causing bleaching.

The exchange of metabolites plays an essential role in the ecological success of the coral–algal symbiosis (Muscatine 1990). Under normal conditions, the symbiont provides necessary PUFAs (C18:4n-3 and C22:5n-3) to the coral host (Papina et al. 2003), C18:4n-3 being a necessary precursor to other n-3 PUFAs (Guil-Guerrero 2007) and C22:5n-3 participating in many biological roles, such as cell signalling regulation and cell proliferation (Salem et al. 2001). However, both FAs were components of the enrichment diet, with C18:4n-3 boosted for D. axifuga and C22:5n-3 for A. millepora at normal temperatures. Yet corals also shape the FA profile of the symbiont by transferring FAs, such as C20:4n-6 and C22:4n-6, which are not typically found in dinoflagellates (Imbs et al. 2014). In the present study, we were not able to separate symbiont FAs from the coral host (due to small coral tissue samples), and most of the specific FA markers that can be used to confirm PUFA exchange between symbiont and coral (Imbs et al. 2014; Tolosa et al. 2011) were present in the diet. Specifically, C22:4n-6 was boosted in D. axifuga fed with the enrichment diet, even though this n-6 PUFA could be synthesized from other n-6 precursors, such as C18:2n-6 and C20:4n-6 that were also present in the diet. Similarly, algal symbiont PUFA biomarkers and the critical FA C22:6n-3 (Bachok et al. 2006; Imbs et al. 2010; Volkman et al. 1998) usually tend to decrease during thermal stress (Kneeland et al. 2013), but instead were increased by the enriched diet. Additionally, the level of C20:5n-3, a major component of the lipid enrichment diet and considered an essential FA to coral–symbiont metabolism (Imbs et al. 2014), was also raised in both species due to its high concentration in the diet. A previous study also demonstrated the increase of coral PUFAs (e.g. C18:4n-3 and C20:4n-6) after the use of Artemia lipid enrichment diet under normal temperatures (Lim et al. 2017). Regardless of the source, elevated proportions of specific PUFAs in both species might enhance thermal stability of membranes and decrease vulnerability to attack by reactive oxygen species (Tchernov et al. 2004).

A considerable amount of work has focused on the assimilation of carbon and nitrogen to elucidate symbiotic contributions (Kopp et al. 2015; Nahon et al. 2013), but more emphasis is needed to understand the functional role of lipids, and the transfer of nutrients from Symbiodinium across the gastroderm (Peng et al. 2011). Only recently have entire genomes become available for some coral species (Shinzato et al. 2011) and their specific Symbiodinium (Lin et al. 2015). With a better understanding of the pathways
contained within each contributing genome and the contribution of a heterotrophic diet, more information on nutrient assimilation can be extracted to better understand lipogenesis, lipid transportation and storage within the symbiotic relationship.

The precise way by which corals obtain lipids may vary among coral colonies (Teece et al. 2011). Overall, the lipid-enriched diet raised the proportions of PUFAs and omega-3 FAs for both coral species (> 20 % for D. axifuga and 13 % for A. millepora at 32 °C, and even more at 26 °C). Yet for A. millepora, PUFA levels were significantly lower at higher temperatures, and can be explained by the dissimilar feeding rates of each species (40 art ml⁻¹ for A. millepora and 90 art ml⁻¹ for D. axifuga). This supports the hypothesis that there will be species-specific winners and losers during recovery from extreme environmental events for corals (Grottoli et al. 2014; Loya et al. 2001), even after using artificial feeding assistance. Nevertheless, the present findings provide a basis to optimise the diet for enhancement of PUFA reserves to increase the resilience of small polyp species, such as the family Acroporidae. Lipid-enriched Artemia may be suitable for large heterotrophic species such as D. axifuga, and the use of smaller zooplankton species, such as the common rotifer Brachionus plicatilis (also suitable for the lipid enrichment process), may be more appropriate for corals with smaller polyps, such as some Acropora species.

Outside of a controlled experimental setting, essential FAs are generated by planktonic sources in the ocean, affecting heterotrophs and food web dynamics (Galloway and Winder 2015). Under larger scale abiotic stress, alterations in planktonic communities are magnified up the food chain (Edwards et al. 2013). When considering the major changes in phyto- and zooplankton communities already detected in the last few decades (Edwards et al. 2013), as well as the predicted shift in planktonic communities to lower nutritional food sources by the effects of ocean climate change (Przytulska et al. 2015), the observed enhancement of coral bleaching resilience through diet might indicate further physiological and ecological implications.

A lipid-enriched Artemia diet increases FA content, algal symbiont density and chlorophyll levels in corals (Lim et al. 2017). In this context, lipid-enriched feeding can be included amongst other novel management approaches currently available to improve the resilience and recovery of coral reefs following bleaching (Hoey et al. 2016; Jin et al. 2016). Considering the geographic extent of many coral reefs, such a dietary intervention has limitations due to the high price of the lipid enrichment diet (~$300 per L) and the associated cost of the application. Nonetheless, it might be considered for small sites with high conservation status or of particular value to the tourism industry.
Overall, this chapter has shown the importance of heterotrophic feeding in coral resilience to bleaching. These findings have significant implications for understanding how rising temperatures may affect species that strongly rely upon non symbiont derived lipids as a source of energy reserves. The lipid-enriched feeding, used as a diet enhancement tool, could improve survival rates during coral relocation in restoration projects, in aquarium trade shipments, and is likely to increase coral growth rates in aquaculture. Furthermore, under predicted stress episodes, the lipid-enriched diet could also be used as a localised solution for targeted endangered species and to improve coral resilience against bleaching.
Chapter 6 - Summary, future work and conclusions
6.1 Summary and future work

The overarching aim of this dissertation was to improve coral aquaculture techniques in order to reduce the necessity for collection of corals from the wild for purposes such as reef restoration and the aquarium trade. The findings from the thesis provide several new insights and approaches that can improve the efficiency and sustainability of the coral aquaculture industry. Furthermore, some of the results are also applicable to coral reef restoration programs. This final chapter provides a summary of the major findings and advances to the field provided within this dissertation. It also places these outcomes in a broader context and makes recommendations for future work to advance the field.

Chapter 1 provided a contextual frame-work of the importance of coral reefs, current threats and a general overview of coral aquaculture. The development of this chapter helped to identify major issues and knowledge gaps in coral aquaculture and restoration as well as a critical revision of previously published coral aquaculture reviews (Barton et al. 2017; Dee et al. 2014; Leal et al. 2016a; Leal et al. 2016b; Murray and Watson 2014; Sheridan et al. 2014).

One of the first gaps identified was the need for a better understanding of the best time to feed corals and their feeding rates, as well as establishing the optimal concentrations. The Artemia feeding concentration used in coral aquaculture research normally is arbitrary (between 0.5 – 4 Artemia ml\(^{-1}\)) (Petersen et al. 2008; Toh et al. 2013a). Chapter 2 focused on answering those questions, using three coral species (Acropora millepora, Hydnophora rigida, Duncanopsammia axifuga) with different biological attributes (e.g. polyp size, feeding strategy and morphology). It was found that corals are able consume higher concentrations of food (> 50 Art ml\(^{-1}\)) and this might have positive implications for coral growth. Optimal feeding regimes are influenced by biological factors, such as polyp size and feeding behaviour. Additionally, contrary to most of the available literature (Borneman and Lowrie 2001; Porter 1974), there were no significant differences between day and night feeding rates. This latter finding, may be useful in a commercial aquaculture context, as it suggests that production costs could be reduced by avoiding the expense of nocturnal feeding without impacting on overall coral feeding rates. Future work in optimizing feeding rates should usefully include an assessment on the suitability of smaller sized food to Artemia, for smaller polyp species. For instance, species in the genus Porites might prefer smaller prey size such as rotifers. Just one climate change study has used rotifers (10 ml\(^{-1}\)) as a source of food 10 ml\(^{-1}\) (Towle et al. 2015), however no evaluation of maximum feeding rate or the effects in
Improving coral aquaculture for reef conservation and the aquarium trade

growth have been investigated. The optimal feeding regimes obtained in this chapter for each of the three coral species investigated were considered in all subsequent chapters.

Having identified the best concentration of food needed to achieve maximum feeding rates for the selected species, in Chapter 3, the next hypothesis related to feeding was to test different diets to find an appropriate one that boosted coral growth. Additionally, due to my personal observation that some corals fragments were discarded due to their lack of polyp (making them unsuitable for the aquarium trade), I decided to test new combinations of cultures to see if there was a use for the fragments without polyps. As part of these tests, I also explored the effects of orientation such as upside-down fixing, an orientation that might happen in the wild due to by wave action (Highsmith 1982). The results showed that fragments without polyps should not be wasted because these corals can produce polyps relatively quickly and contribute to production. The culturing of waste fragments within aquaculture will lead to a reduction of coral waste during the fragmentation process and improvements in the efficiency of the industry. Furthermore, the upside-down orientation culture method was found to increase polyp production in large polyp species. This finding should be further explored by testing the use of fragments without polyps in other commercially valuable species. Further trials should be considered on similar morphotypes within species in the genera Cladocora, Caulastrea, Euphyllia, Eusmillia, and even the cold deep-water species such as Lophelia pertusa.

Inspired by the results related to the upside-down technique developed in the previous chapter, in Chapter 4, I tested the effects of the same method, but in small polyp species, as a tool to increase the self-attachment speed. The results, demonstrated that corals fixed upside-down self-attached faster, in higher numbers, and grew significantly wider over the substrate. This chapter has implications for coral restoration attempts using fragments, as it can help reduce coral mortality associated with self-attachment failure. However, because all experiments were developed ex situ, forthcoming research around this chapter should test the effectiveness of this method in the field, as well as on other similar branching small polyp species, such as the genera Acropora, commonly used in coral reef restoration (Chamberland et al. 2015; Forrester et al. 2014; O'Donnell et al. 2017). Long term survival comparisons between upside-down and right way up fragments translocated to the wild will improve understanding of the efficiency of this method for reef restoration applications.

The observation that corals that survive bleaching events have larger lipids reserves (Grottoli and Rodrigues 2011; Grottoli et al. 2006; Rodrigues and Grottoli 2007)
was the impetus for the investigations in Chapter 5. Here, I examined the effectiveness of using a lipid enrichment diet as a tool to prevent bleaching episodes caused by temperature stress. I found that corals are able to increase their lipid reserves through a diet based on high levels of omega-3 fatty acids, and that this increased resilience against temperature stressors. Although I acknowledge that the findings of this chapter might have limitations to be used in extensive areas of corals reefs, it is also well known that in aquaculture, coral bleaching can be regularly triggered by stressors such as the fragmentation process, fluctuating salinity, nutrient and culture conditions, as well as transportation and shipping. Providing a lipid enriched diet before fragmentation and transport might help to reduce mortality and improve long-term survivorship of the cultured corals. At the same time, the process can be adopted to protect endangered coral species or build resilience in mariculture systems before predicted temperature stress. Probably at the same time that this chapter was being developed, another team of researchers from Malaysia had a similar idea of testing the effects of a lipid enrichment diet in another species (*Galaxea fascicularis*), but without the increasing temperature factor. Similar to my results, they demonstrated positive effects on the health of the coral after consuming the diet (Lim et al. 2017). In a broader context, procedures that reduce coral mortality on aquaculture farms and in the aquarium trade, like the ones investigated here, will indirectly reduce wild coral collection demand.

In light of the overall success of the lipid enrichment diet to enhance coral resilience against bleaching events, further research should focus on the following questions:

- It is possible to administer a lipid-enriched diet by dissolving the lipid emulsion straight into the water without the use of *Artemia*?
- How many days of continuous lipid-enrichment feeding are necessary to achieve the maximum levels of lipids in the corals?
- How long do corals have enhanced lipid reserves once the lipid-enriched diet is discontinued?

Moreover, due to the current use of coral sexual reproduction in restoration projects (Baria et al. 2012; dela Cruz and Harrison 2017), future research involving the lipid enriched diet and its effect on sexual reproduction might be considered around the following research questions:

- Does a lipid-enriched diet also enhance coral sexual reproduction?
- Does the lipid-enriched diet transfer into the gametes or larvae?
Do larvae from corals that were fed with a lipid-enriched diet have better rates of settlement and survival?

All experiments performed in this thesis used plastic vials of 150 ml, therefore, as a future consideration, the possibility of scaling up the methodologies and techniques for future commercial coral aquaculture scenarios warrant additional testing (e.g. the effects of stronger water flow, larger tanks, several species in the same tank, etc.). Furthermore, recent research has demonstrated that phenotype/genotypes selection might produce significant effects on the success of the cultured species (Lohr and Patterson 2017; O'Donnell et al. 2017). The inclusion of these factors in future experiments might be another direction to follow to improve coral aquaculture research.

Finally, two new promising frontiers might stimulate coral aquaculture research in the future: (1) corals fulfil all the requirements as material for osteoplasty and 3D–scaffolds of human bone tissue and human bone revitalization (Green et al. 2017; Popov et al. 2015; Sergeeva et al. 2014); and (2) the culture of coral microbial communities as a source of bioactive compounds for drug discovery (Leal et al. 2013; Pimentel et al. 2016). Both research areas will need coral aquaculture to supply high quantities of coral to keep going with the associated necessary research, and once all the technology is completely validated, there will be a need for corals as a main material to replace bones. Because of the importance of the successful medical application, coral aquaculture is likely to receive more attention and investment in the future.

6.2 Conclusion

This dissertation provides new information and techniques that may improve coral aquaculture for coral restoration and the aquarium trade. Furthermore, the three species studied (Acropora millepora, Hydnophora rigida and Duncanopsammia axifuga) can be considered as suitable species for coral aquaculture because they have high survival after the fragmentation process and increased growth rates with Artemia feeding. In coming years, the work in my thesis on the concentration of food to achieve maximum feeding rates on the studied and similar species, the use of waste fragments without polyps, the upside-down orientation to accelerate polyp production and coral attachments and the use of a lipid-enriched diet to enhance coral resilience may well be implemented and regularly used by coral reef researchers and the aquaculture industry.
References


References


References


References


References


Appendices
Appendix 1. Methods to measure increments in weight or length of live corals

The use of non-destructive methods is required to measure coral growth in coral aquaculture facilities. Below is a brief description of the most commonly used techniques to measure increments in weight or length, without the necessity of sacrificing corals. These methods are relatively simple to carry out routinely, compared with the methods used to estimate surface area or increments in calcification.

**Buoyant weight:** This method was developed more than 40 years ago (Bak 1973) and has since undergone some modifications (Davies 1989; Herler and Dirnwöber 2011). It allows the researcher to estimate the percentage daily coral growth (% CG day\(^{-1}\)) by using the formula: \((% \text{ CG day}^{-1}) = \frac{(wf - wi)}{\Delta T} \times 100\); where \(wf\) and \(wi\) are the final and initial weights, and \(\Delta T\) is the time interval (days) between these measurements. This method is limited to colonies that can be detached (Herler and Dirnwöber 2011) and the density of the water also needs to be carefully considered. Despite these limitations, the buoyant weight method is one of the most accepted methods in coral experimentation (e.g. Davies 1989; Holcomb et al. 2010; Movilla et al. 2012; Osinga et al. 2011; Piniak and Brown 2008; Towle et al. 2015).

**Ecological volume:** This method, developed by Rinkevich and Loya (1983), measures the total volume occupied by a coral colony, including the inner spaces between branches, and approximates the shape of the coral to a cylinder or sphere, depending on the colony form. Basically, it is calculated by measuring the height (\(h\)), width (\(w\)) and length (\(l\)) of the coral and then approximating the shape to a cylinder using the formula:

\[ V = \pi r^2 h, \text{ in which } r = \frac{l + w}{4} \]

Although the method has been used in several experiments (Levy et al. 2010; Shaish et al. 2010; Bongiorni et al. 2011; Osinga et al. 2011; Shafir et al. 2006a; Toh et al. 2013b), it tends to provide a relatively imprecise approximation of growth.

**Drip-dry weight or wet weight:** Here the weight of each fragment (outside of the water) is determined by using a balance, after cleaning the excess water, allowing the calculation of growth rate within a set period. Coral growth is obtained by the change in weight over time, and the specific growth rate (SGR) is obtained using a similar formula to the buoyant weight:

\[ \text{SGR (day}^{-1}) = \frac{\ln Wt - \ln Wt - 1}{\Delta T} \]

This method has also been used in several studies (Schlacher et al. 2007; Wijgerde et al. 2011; Wijgerde et al. 2012).
Appendices

**Basal proportion:** This method measures the smallest and largest diameter of the basal portion of each coral fragment, to determine an average. Coral growth is then measured by calculating the difference between the final and initial diameters (Green et al. 2010).

**Total linear extension:** An estimate of growth is obtained by measuring the sum of the linear length of all of the live branches of a colony, subtracting the initial size from the final size and then dividing by the number of days. This is often done by analysing pictures to measure the size of the branches with a scale as reference (Mercado-Molina et al. 2015; Mercado-Molina et al. 2014). It has been suggested that skeletal extension rates do not represent an accurate method for measuring growth since many colonies can grow more around the base than in the branches (Schlacher et al. 2007). Nevertheless, the method is well accepted and has been used as a growth measurement technique (Lohr and Patterson 2017).

**Linear increment in height:** This represents the linear dimension measured from the top to the bottom of a colony (Gomez et al. 2014). As mentioned previously with the linear extension method, this method can underestimate coral growth at the base (Schlacher et al. 2007).

**Geometric mean diameter (d):** The greatest and least diameter (GD / LD) of the live sections of a colony are measured, and the final value is calculated with the following formula:

\[ D = \sqrt{((GD \times LD))} \] (Clark and Edwards, 1995; Toh et al. 2013b).

**Colony radius:** This method represents a slight variation in the geometric mean diameter method above, and it is derived from the longest diameter (L) and the longest perpendicular dimension of this (W), using the formula: \( \frac{1}{2} \) SQR root \((L \times W)\) (Gomez et al. 2014).
### Appendix 2. Non-enriched *Artemia* vs. lipid-enriched *Artemia* fatty acid profiles.

<table>
<thead>
<tr>
<th>Ret. Time</th>
<th>Fatty acids</th>
<th>Common Name</th>
<th>Normal Artemia Mean (% A)</th>
<th>SD</th>
<th>Enriched Artemia Mean (% A)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.654</td>
<td>C14:0</td>
<td>Myristic</td>
<td>0.07</td>
<td>0.01</td>
<td>1.44</td>
<td>0.26</td>
</tr>
<tr>
<td>24.252</td>
<td>C14:1</td>
<td>Myristoleic</td>
<td>0.04</td>
<td>0.00</td>
<td>0.09</td>
<td>0.01</td>
</tr>
<tr>
<td>26.788</td>
<td>C16:0</td>
<td>Palmitic</td>
<td>1.01</td>
<td>0.22</td>
<td>6.67</td>
<td>1.07</td>
</tr>
<tr>
<td>27.796</td>
<td>C16:1n-7</td>
<td>Palmitoleic</td>
<td>0.22</td>
<td>0.06</td>
<td>0.56</td>
<td>0.09</td>
</tr>
<tr>
<td>29.159</td>
<td>C17:0</td>
<td>Magaric</td>
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<td>0.01</td>
<td>0.13</td>
<td>0.02</td>
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<td>Stearic</td>
<td>0.63</td>
<td>0.14</td>
<td>1.78</td>
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<td>32.295</td>
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<td>Oleic</td>
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<td>0.38</td>
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<td>32.472</td>
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<td><em>cis</em>-Vaccenic</td>
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<td>35.48</td>
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<td>0.37</td>
<td>5.35</td>
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<tr>
<td>35.744</td>
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<td>36.526</td>
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<td>11-Eicosenoic</td>
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<td>37.911</td>
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<td>8,11,14-Eicosatrienoic</td>
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<td>0.00</td>
<td>10.94</td>
<td>2.65</td>
</tr>
</tbody>
</table>
### Appendices

#### Appendix 3. Mean (± SE) fatty acid profile for *D. axifuga*.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Trivial name</th>
<th>Ret.Time</th>
<th>Unfed 26 °C</th>
<th>Normal Art. 26 °C</th>
<th>Enriched Art. 26 °C</th>
<th>Unfed 32 °C</th>
<th>Normal Art. 32 °C</th>
<th>Enriched Art. 32 °C</th>
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<tr>
<td></td>
<td></td>
<td>(min)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
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<tr>
<td><strong>Saturated fatty acid (SFA)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>2.42</td>
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<td>23.82</td>
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<td>1.79</td>
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<td>8.52</td>
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<td>8.15</td>
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<td>0.77</td>
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<td><strong>Polyunsaturated (PUFA)</strong></td>
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<td>C18:3n-3</td>
<td>α-Linolenic (ALA)</td>
<td>37.69</td>
<td>0.16</td>
<td>0.08</td>
<td>0.31</td>
<td>0.09</td>
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<td>0.02</td>
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<td></td>
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<td>C20:5n-3</td>
<td>Eicosapentaenoic (EPA)</td>
<td>43.3</td>
<td>3.20</td>
<td>0.16</td>
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<tr>
<td>C22:5n-3</td>
<td>Docosapentaenoic (DPA)</td>
<td>47.25</td>
<td>3.01</td>
<td>0.32</td>
<td>3.67</td>
<td>0.15</td>
<td>4.01</td>
<td>0.16</td>
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<td>Docosahexaenoic (DHA)</td>
<td>47.71</td>
<td>9.27</td>
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<tr>
<td>C18:2n-6</td>
<td>Linoleic (LA)</td>
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<td>1.24</td>
<td>0.21</td>
<td>1.90</td>
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<tr>
<td>C18:3n-6</td>
<td>Gamma-Linolenic</td>
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<td>0.08</td>
<td>4.26</td>
<td>0.33</td>
<td>4.22</td>
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<td>Dihomo-gamma Linolenic</td>
<td></td>
<td>39.78</td>
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<td>0.05</td>
<td>0.54</td>
<td>0.04</td>
<td>0.44</td>
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<td>Arachidonic</td>
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<td>41.69</td>
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<td>8.90</td>
<td>0.22</td>
<td>8.94</td>
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<td>Docosatetraenoic</td>
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<td>5.10</td>
<td>0.11</td>
<td>5.91</td>
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<tr>
<td>C20:2n-6</td>
<td>11,14-Eicosadienoate</td>
<td></td>
<td>40.28</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>0.05</td>
<td>0.57</td>
</tr>
<tr>
<td>C20:2</td>
<td>Eicosadienoic</td>
<td></td>
<td>41.13</td>
<td>1.65</td>
<td>0.07</td>
<td>1.24</td>
<td>0.08</td>
<td>1.35</td>
</tr>
</tbody>
</table>

**Mean** and **SE** values are provided for comparison.
### Appendix 4. Mean (± SE) fatty acid profile for *Acropora millepora*.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Trivial Name</th>
<th>Ret.Time (min)</th>
<th>Normal Art. 26 °C</th>
<th>Unfed 26 °C</th>
<th>Enriched Art. 32 °C</th>
<th>Unfed 32 °C</th>
<th>Normal Art. 32 °C</th>
<th>Enriched Art. 32 °C</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
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<td><strong>Saturated fatty acid (SFA)</strong></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>C14:0</td>
<td>Myristic</td>
<td></td>
<td>23.89</td>
<td>1.70</td>
<td>3.18</td>
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<td>3.17</td>
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<tr>
<td>C16:0</td>
<td>Palmitic</td>
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<td>29.11</td>
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<td>0.53</td>
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<tr>
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<td>Stearic</td>
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<td>2.21</td>
<td>11.51</td>
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<td>Arachidic</td>
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<td></td>
<td>0.93</td>
<td>0.10</td>
<td>0.87</td>
<td>0.05</td>
</tr>
</tbody>
</table>

| Monounsaturated (MUFA) |            |                |       |    |       |    |       |    |       |    |       |    |
| C14:1      | Myristoleic  |                | 25.27 |       | 0.47 | 0.02 | 0.51 | 0.05 | 0.64 | 0.11 | 0.45 | 0.03 |
| C16:1n-7  | Palmitoleic  |                | 30.09 |       | 2.41 | 0.21 | 2.26 | 0.08 | 2.51 | 0.16 | 2.18 | 0.41 |
| C18:1n-9  | Oleic        |                | 34.61 |       | 7.32 | 1.46 | 4.68 | 0.07 | 4.82 | 0.58 | 7.36 | 0.87 |
| C18:1n-7  | cis-Vaccenic |                | 34.81 |       | 0.65 | 0.14 | 0.17 | 0.10 | 0.41 | 0.06 | 0.38 | 0.27 |
| C20:1n-9  | Eicosenoic   |                | 38.94 |       | 2.55 | 0.57 | 1.80 | 0.10 | 1.80 | 0.09 | 1.58 | 0.37 |

| Polyunsaturated (PUFA) |            |                |       |    |       |    |       |    |       |    |       |    |
| C18:2n-6  | Linoleic (LA)|                | 36.01 |       | 1.65 | 0.23 | 2.36 | 0.84 | 1.48 | 0.21 | 2.67 | 0.68 |
| C18:3n-6  | Gamma-linolenic |            | 36.91 |       | 3.73 | 0.32 | 4.67 | 0.27 | 4.29 | 0.54 | 3.33 | 1.31 |
| C20:3n-6  | Dihomo-gamma Linolenic | | 39.78 |       | 0.56 | 0.06 | 0.67 | 0.06 | 0.86 | 0.06 | 0.17 | 0.10 |
| C20:4n-6  | Arachidonic  |                | 41.69 |       | 6.02 | 0.45 | 7.14 | 0.66 | 6.73 | 0.52 | 5.59 | 1.04 |
| C22:4n-6  | Docosatetraenoic |        | 45.73 |       | 3.97 | 0.34 | 4.72 | 0.38 | 4.58 | 0.28 | 4.00 | 0.90 |
| C20:2n-6  | 11,14-Eicosadienoic | | 40.28 |       | 0.36 | 0.02 | 0.26 | 0.09 | 0.26 | 0.09 | 0.28 | 0.16 |
| C20:2     | Eicosadienoic |                | 41.13 |       | 0.65 | 0.06 | 0.76 | 0.05 | 0.71 | 0.09 | 0.58 | 0.18 |