Manipulation of coral photosymbionts for enhancing resilience to environmental change

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Statement of the contribution of others

This thesis includes collaborative work with my advisors Madeleine van Oppen, Mia Hoogenboom and Bette Willis. While undertaking these collaborations I was responsible for experimental design, data collection, analysis, and interpretation of my results. My co-authors provided intellectual guidance, editorial assistance and financial support.

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General Abstract

Ocean warming is occurring at an unprecedented rate. Only a small increase in seawater temperature can disrupt the symbiotic relationship between corals and their photosynthetic algae causing coral bleaching. The bleaching threshold of corals is largely dependent on the microalgae they host. Some studies have indicated the ability for small increases in corals' tolerance to environmental change through shifts in their symbiont communities. However, the increase in frequency of severe bleaching events that have led to worldwide loss of coral cover indicate that this is not enough for coral persistence. This thesis investigates the feasibility and efficacy of manipulating algal symbiont populations associated with corals to enhance their stress tolerance in an era of rapid environmental change.

Due to their comparatively short generation time, coral algal symbionts have the potential to evolve more rapidly to environmental changes than their coral host. **Chapter two** investigates the thermal tolerance of the most common algal symbiont of Great Barrier Reef corals, *Cladocopium goreaui*, after ~80 asexual generations (2.5 years) of *in vitro* directed laboratory evolution at an elevated temperature. Using a reciprocal transplant design, I show that the upper temperature tolerance range of the selected *C. goreaui* increased, evidenced by superior photophysiological performance, growth rates and lower levels of extracellular reactive oxygen species, relative to wild-type cells. In comparison, wild-type *C. goreaui* cells were unable to photosynthesise or grow at elevated temperature. The enhanced thermal tolerance of the selected *C. goreaui in hospite* was less apparent. Two of three coral species tested showed positive growth when harbouring the selected *C. goreaui* at elevated temperature, compared to those that hosted the wild-type cells that had negative growth at elevated temperature. Despite this, recruits of the three coral species bleached regardless of whether they hosted the thermally selected or the wild-type *C. goreaui.*

Important next steps were to decipher the genetic basis underlying enhanced thermal tolerance in the selected algal *C. goreaui*. Therefore, **Chapter three** investigates the differences in gene expression between the wild-type and selected cells during the reciprocal transplant experiment. Samples were taken at three time points over 35 days and a *de novo* transcriptome was assembled. Comparative transcriptomics revealed significant differences in gene expression between the wild-type and selected *C. goreaui* at elevated temperature. The wild type cells displayed an unstable transcriptomic response of upregulated genes over time, involving large changes in the numbers of genes upregulated and their associated functions. Down-regulated genes, however, were

consistently photosynthesis-related, concurrent with their inferior photosynthetic performance at elevated temperature as detailed in chapter 2. The thermally selected *C. goreaui* shared very few differentially expressed genes with the wild-type cells, having a more stable transcriptomic response to elevated temperature over time. Upregulated genes largely involved those encoding DNA transcription and initiation processes. Although some photosynthetic-related genes were downregulated during one time point, the majority of downregulated genes were involved in the regulation of cell projection organisation.

Chapters two and three investigate the most common Great Barrier Reef species of coral photosymbiont, *C. goreaui.* However, the family that they belong to, the Symbiodiniaceae, is genetically diverse and studies have found wide phenotypic differences between species, with differing thermal tolerances. This led me to testing whether thermal selection experiments could be used successfully across a range of species in the Symbiodiniaceae. Therefore, in **chapter four** I examine the response of five genetically distinct strains of the Symbiodiniaceae, belonging to four genera, over the course of approximately one year. For three genera I observed a stable adaptive change after only 41-69 asexual generations, where selected cells grew faster and in some cases had higher photosynthetic efficiencies than their wild-type counterparts at elevated temperature. The observed increases in growth rates are comparable with evolutionary experiments in other microalgae, where thermally selected populations have been exposed to elevated temperatures for up to 400 generations.

The Symbiodiniaceae are not the only algae to be associated with corals. Apicomplexan-like microalgae were discovered in 2008 and the phylum Chromerida was created. Chromerids have been isolated from corals and contain a functional photosynthetic plastid. Their discovery opens a new avenue of research into the use of alternative/additional photosymbionts of corals. Furthermore, not only do global environmental changes pose a threat to marine organisms but also the simultaneous effects of local stressors such as herbicide additions to coastal systems that often coincide with high summer temperatures. Diuron is one of the most commonly applied herbicides in the catchments of the Great Barrier Reef, acts to inhibit photosynthesis in plants and algae and has been directly linked to coral bleaching. In **chapter 5** I test the performance of four chromerid populations as well as *C. goreaui* in response to elevated temperature, diuron and their combined exposure. Three of the four chromerid strains exhibited high thermal tolerances and two exceptional herbicide tolerances, greater than any observed for photosynthetic microalgae. I subsequently investigate the ability of the chromerids to form a symbiosis with larvae of two common GBR coral

species under ambient and stress conditions. Chromerid uptake by coral larvae was low compared to *C. goreaui*. I did not observe any overall negative or positive larval fitness effects of the infection with chromerid algae vs. *C. goreaui*. However, the possibility that chromerid algae may have more important roles in later coral life stages or with other species of coral cannot be excluded.

The research presented in this thesis is among the first to test the possibility of experimental evolution to enhance the thermal tolerance of coral symbionts belonging to the Symbiodiniaceae, as well as the use of the potentially alternative symbionts, the chromerids. My results show that it is possible to experimentally evolve cultured Symbiodiniaceae strains across multiple species and highlight the genetic and molecular pathways that underpin thermal tolerance in the most common Great Barrier Reef species, *C. goreaui*. Despite increased thermal tolerance of the thermally-selected Symbiodiniaceae *in vitro* and the high thermal and diuron tolerance of some chromerid populations, these were unable to significantly enhance the upper thermal limit or diuron tolerance of the coral-algal symbiosis. Therefore, further work into the algal-coral association and bleaching response is required to assess whether algal symbiont manipulation has the potential to be a valuable tool in coral reef conservation and restoration initiatives in a rapidly changing ocean.

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Chapter 1: General Introduction

Global warming and coral reefs

Global warming is occurring at a rate not experienced on Earth for at least 50 million years (Hönisch et al. 2012; Zeebe et al. 2014). The cause is the anthropogenic release of vast quantities of atmospheric carbon dioxide (CO_2) and other greenhouse gasses into the atmosphere. Since the industrial revolution, CO_2 concentrations have increased by 45% (www.esrl.noaa.gov/gmd/ccgg/trends/) and consequently a modest global warming of ~0.92°C has been observed (1880-2017) (Lough, Anderson, & Hughes, 2018). The world's nine hottest years have occurred since 2005, with 2015, 2016 and 2017 being the warmest years on record (World Meterological Organization 2018). Tropical sea surface temperatures (SSTs) have largely tracked that of global average atmospheric temperatures (Lough et al., 2018). This unprecedented rate of warming is considered too rapid for many marine organisms to keep up, leading to the prediction of mass species extinction by 2100 (Dawson, Jackson, Joanna, Prentice Iain C, & Mace, 2011; Hoegh-Guldberg & Bruno, 2010; Pacifici et al., 2015; Pereira et al., 2010; Urban, 2015). Heating to date has had devastating consequences for the world's tropical coral reefs that are already existing at their upper thermal limits (Hoegh-Guldberg et al., 2008). An increase of only 1°C above mean summer temperatures can cause the loss of corals' obligate symbionts (coral bleaching), ultimately resulting in coral death. Rising ocean temperatures have triggered three global bleaching events (1998, 2010 and 2014-2017) since records began in the 1980s (Hughes et al. 2017a). Not only have bleaching events become more extreme and widespread, but the time between successive events has declined by 4.6-fold since the early 1980s (Hughes et al., 2017). As a consequence, narrow time windows do not allow full coral recovery (Hughes et al., 2018). Anthropogenic carbon emissions that are driving ocean warming are unlikely to be curbed, and SSTs are expected to be 2-3 °C higher by the end of the century, compared to pre-industrial revolution temperatures (Caldeira and Wickett 2003; Collins et al. 2013). To preserve just 10% of the world's coral reefs, it has been predicted that warming must be limited to below 1.5°C (Frieler et al. 2013).

The importance of coral reefs

Tropical coral reefs are an incredibly valuable ecosystem (Cesar et al. 2003; Brander et al. 2007). Fuelled by their algal symbiosis, reef-building corals produce complex three-dimensional calcium carbonate structures and thus a habitat of high heterogeneity that provides a multitude of niches for a variety of organisms. Furthermore, coral reefs link other ecosystems such as mangroves, sea grass beds and the open ocean through harbouring species that move between them in search for

breeding, feeding or nursery grounds (Cocheret de la Morinière et al. 2003; Mumby et al. 2004). As a result, the biodiversity of coral reefs exceeds that of any other ecosystem on Earth, at the level of the Phyla (Porter and Tougas 2001). At the species level, there are estimates that coral reefs support up to nine million species worldwide with only an estimated 10 % of species having been described (Knowlton 2001). Among known species, coral reefs support over 4000 species of fish, 700 species of coral and thousands of other forms of life, including microbial species (Cesar et al. 2003). In addition to their high biodiversity, coral reefs are one of the most productive ecosystems on Earth (e.g. Odum and Odum 1955, Connell 1978). Millions of people's lives depend on the natural resources that are provided by coral reefs through food and income (Costanza et al. 1997; de Groot et al. 2012). Over US$20 trillion of ecosystem goods and services are provided to the world economy from coral reefs (Costanza et al. 1997; de Groot et al. 2012) that include fisheries, coastal protection , tourism/recreation and as well as invaluable aesthetic and cultural benefits (Spurgeon 1992; Peterson and Lubchenco 1997; Cesar et al. 2003; Brander et al. 2007; Spalding et al. 2017)

With global climate change and reef degradation come the loss of biodiversity and physical structure.. A recent model projected a loss of US$1.37 to 5.7 billion annually in coral value due to climate change based on the International Panel of Climate Change's (IPCC) projected representative concentration pathways (Chen, Chen, Chu, & McCarl, 2015).

The Great Barrier Reef (GBR) is the world's largest coral reef ecosystem and was inscribed on the World Heritage list in 1981. Spanning an area of more than 344 000 km^2, more than 2000 km in length and containing ~3000 coral reefs, the GBR contains a high diversity of marine life. It fuels fisheries and tourism activities, provides vast stretches of coastal protection and its economic value to the Australian economy is estimated at $56 billion with an economic contribution of $6.4 billion *per* year (Deloitte Access Economics 2017).

The coral-algal symbiosis

Reef-building corals build extensive, complex structures in shallow, oligotrophic waters. These feats of engineering are powered by photosynthesis of the algal endosymbionts (Roth 2014). Dinoflagellate microalgae belonging to the family Symbiodiniaceae can occur in high densities of over one million cells *per* square centimetre of coral host surface area (Mieog, van Oppen, Berkelmans, Stam, & Olsen, 2009). Corals form an obligate symbiosis with the Symbiodiniaceae (LaJeunesse et al., 2018) by providing over 90% of the corals' energy through the translocation of photosynthetic carbon (Muscatine and Porter 1977; Falkowski et al. 1984; Muscatine 1990). This

fixed carbon is used by the coral host to fuel growth and calcification (Goreau, 1959; Muscatine, 1990). In return, the Symbiodiniaceae gain refuge and nutrients from the coral host. While some brooding coral species pass their Symbiodiniaceae vertically to the subsequent generation through their eggs, most corals on the Great Barrier Reef are broadcast spawners, over 80% of which produce aposymbiotic (lack symbiotic microalgae) gametes (Baird, Guest, & Willis, 2009). Aposymbiotic larvae and/or recruits acquire microalgae horizontally from free-living populations in the surrounding water column or sediment and the Symbiodiniaceae become established within the endodermal cells of the host (Baird et al., 2009).

The Symbiodiniaceae are genetically diverse (LaJeunesse et al., 2018). Until recently, these algae were classified in a single genus, *Symbiodinium*, which was comprised of nine phylogenetic clades (A toI; Pochon and Gates 2010) with each clade consisting of multiple types (e.g. van Oppen et al. 2005a). More recently, the classification of these evolutionarily divergent clades has been revised with clades now described as equivalent to genera in the family Symbiodiniaceae (LaJeunesse et al., 2018). With 22 species formally classified, hundreds more are likely to be candidates for species classification with further investigation into their diversity (Thornhill et al. 2014). Corals can host multiple genetic variants of the Symbiodiniaceae simultaneously (Boulotte et al., 2016; Mieog, van Oppen, Cantin, Stam, & Olsen, 2007; Silverstein, Correa, & Baker, 2012) and dominant Symbiodiniaceae types *in hospite* can vary in relative abundance, depending on coral life-stage and environmental conditions (e.g. Little et al., 2004).

With genetic diversity comes physiological, behavioural and morphological diversity in the Symbiodiniaceae. For example, a *Symbiodinium* species (formerly *Symbiodinium* ITS2 clade A) have been associated with the fast colonisation of health-compromised corals (Toller et al. 2001), low transfer of photosynthate (Stat, Morris, & Gates, 2008) and high competitive ability in mixed *in vitro* cultures (Rowan, 1998). *Cladocopium goreaui* (formerly *Symbiodinium* ITS2 type C1) had 87% greater electron transport rates of photosystem II than a species of *Durusdinium* (formerly *Symbiodinium* ITS2 type D1) *in hospite* (Cantin, van Oppen, Willis, Mieog, & Negri, 2009) and corals harbouring *Cladocopium* sp. (clade C) grew up to three times as fast as those harbouring *Durusdinium* sp. (clade D) (Little et al. 2004). Importantly, under adverse conditions, genetic variants of the Symbiodiniaceae may differ in their responses; two populations of *C. goreaui* differed in their sensitivity to thermal stress both *in vitro* (Levin et al. 2016) and *in hospite* (Howells et al., 2012), despite belonging to the same species. Furthermore, the association with different species in the Symbiodiniaceae may alter the thermal stress tolerance of the coral holobiont (Abrego, van Oppen,

& Willis, 2009; Berkelmans & van Oppen, 2006; Howells et al., 2012; Mieog et al., 2009; Sampayo, Ridgway, Bongaerts, & Hoegh-Guldberg, 2008; Stat & Gates, 2011). For example *Durusdinium* spp. have often been found to dominate corals on reefs that are exposed to heat stress or have had a history of bleaching (Baker, Starger, McClanahan, & Glynn, 2004; Glynn, Maté, Baker, & Calderón, 2001; Jones, Berkelmans, van Oppen, Mieog, & Sinclair, 2008; LaJeunesse, Smith, Finney, & Oxenford, 2009; Rowan, 2004; Toller et al., 2001; van Oppen, Mahiny, & Done, 2005) and have been shown to enhance the thermal tolerance of the coral *Acropora millepora,* a common Indo-Pacific species, by 1-1.5 °C through the shuffling of symbiont types (Berkelmans & van Oppen, 2006). It is clear that the physiological tolerance levels of the Symbiodiniaceae that a coral hosts can be as significant as the coral host in determining holobiont physiological performance under adverse conditions.

Reef-building corals consist not only of the cnidarian animal and the Symbiodiniaceae, but a diverse suite of other microorganisms that form an association with the coral host. Thus, the coral microbiome also includes bacteria, viruses, archaea, fungi, and other micro-eukaryotes (Rohwer et al. 2002; Blackall et al. 2015). Although the function of many of these taxa is unknown, the coral microbiome is thought to be an important factor in maintaining coral health (Bourne et al. 2016). In addition to the Symbiodiniaceae, there is a diversity of eukaryotes associated with corals that include the apicomplexan-related lineages , many of which are largely uncharacterised (Clerissi et al. 2018), but can occur in high prevalence across coral groups (Kirk et al. 2013b, a; Kwong et al. 2018). Apicomplexan-like algae with a functional photosynthetic plastid were found associated with corals that make up the Chromerida and currently consist of two described species, *Chromera velia* (Moore et al. 2008) and *Vitrella brassicaformis* (Oborník et al. 2012); both were isolated from Great Barrier Reef corals. Only two experiments to date have investigated the symbiosis of the chromerids with corals. Three species of *C. velia* were introduced to aposymbiotic coral larvae and were taken up and hosted within endodermal cells after sampling one and three days later (Cumbo et al., 2013). Another chromerid species was shown to induce a classic transcriptomic parasitic response in coral larvae 24 hours after inoculation (Mohamed et al., 2018). Despite the prevalence of apicocomplexan-related lineagess associated with corals, their relative contribution to the coral-algal symbiosis as an alternative/additional source of phototrophic energy is still unknown. Furthermore, nothing is known about their environmental tolerances to adverse conditions such as ocean warming.

Effects of ocean warming on the Symbiodiniaceae

During photosynthesis, absorbed sunlight drives the photochemical reactions of photosynthesis. However, experiments have shown that only 4% of absorbed light is required for photosynthesis (Brodersen et al. 2014). Thus, excess light is re-emitted as chlorophyll fluorescence or dissipated as heat, processes known as non-photochemical quenching (NPQ). When heat is applied, in addition to excess light, there are multiple impacts to organismal physiology. Reactive oxygen species (ROS) are produced as a by-product of photosynthesis that can cause damage to the photosynthetic apparatus that must be repaired (Murata et al. 2007). If the rate of damage exceeds the rate of repair, photosynthetic efficacy is reduced and this results in photoinhibition (Niyogi 1999). In addition to NPQ capacity, various photoprotective processes exist to combat photoinhibition including antioxidant pathways, photorespiration, protein repair and synthesis (Niyogi 1999). Photochemical reactions and photosynthetic membranes are also sensitive to temperature changes. Elevated temperature can affect the rates of repair of the photosynthetic proteins, alter thylakoid membrane fluidity and contribute to the uncoupling of the photosystems (Takahashi, Nakamura, Sakamizu, Woesik, & Yamasaki, 2004; Tchernov et al., 2004; Warner, Fitt, & Schmidt, 1999). Furthermore, high levels of ROS can disrupt photosynthetic machinery by damaging lipids, proteins, DNA and photosynthetic membranes (Lesser, 2006; Murata et al., 2007; Tchernov et al., 2004). Exposure to even sub-lethal elevated temperature is known to cause a breakdown in the photosynthetic machinery of microalgae. As a consequence, the fixation of CO_2 becomes limited (Murata et al. 2007) thus negatively affecting microalgal cell growth.

Growth kinetics and photobiology differ within the Symbiodiniaceae. Many studies suggest that different species of the Symbiodiniaceae have different physiological thermal optima that affect their ability to respond to environmental changes (Klueter et al. 2017). For example, growth and photosynthetic efficiencies were unaffected by two weeks at elevated temperature (33°C) in a *Fugacium* sp. (formerly *Symbiodinium* ITS2 type F1) accompanied by increases in antioxidant defence enzymes (Krueger et al. 2014). *Cladocopium goreaui* and a *Breviolum sp.* (formerly *Symbiodinium* C1 and B1) had greater ROS levels at elevated temperature, while ROS levels did not increase for a *Symbiodinium* sp. and *Fugacium sp.* (formerly *Symbiodinium* ITS2 types A1 and F1) at elevated temperature (McGinty, Pieczonka, & Mydlarz, 2012). Two populations of *C. goreaui* showed either a decrease in photosynthetic efficiency coupled with an increase in ROS or no change in either (Levin et al. 2016). A *Breviolum* sp. had increase ROS levels at 32 °C, while a *Symbiodinium* sp. did not (Suggett et al. 2008). The leakage of ROS into the cell's surrounding environment has been linked to triggering the bleaching response of corals (Downs et al., 2002; Krueger et al., 2015;

Lesser, 2006; Warner et al., 1999). Additionally, a disruption in photosynthesis and reduction in photosynthetically fixed carbon produced and translocated to the coral host can lead to negative downstream consequences for the coral (Baker, Andras, Jordán-Garza, & Fogel, 2013; Cantin et al., 2009; Szmant & Gassman, 1990; Wiedenmann et al., 2013; Wooldridge, 2009a) that may also induce a bleaching response.

While the physiological stress response of the Symbiodiniaceae is well studied, the molecular pathways underlying their thermal stress responses are less known. A limited transcriptomic response to heat stress has often been observed for the Symbiodiniaceae with a majority of studies showing less than two-fold expression values (Gierz et al. 2017), or no significant difference in expression between populations at control and elevated temperatures (Barshis et al. 2014). Gierz et al. (2017) identified genes in the transcriptome of a *Fugacium* sp. (formerly *Symbiodinium* ITS2 clade F) exposed to 30–31.5°C that were involved in the antioxidant network, heat shock proteins and transcription factors, DNA damage repair, proteasomal degradation pathways, apoptosis, photosynthesis as well as metabolism and growth; processes that are usually upregulated as a typical cellular response to stress (Martindale and Holbrook 2002; Kültz 2005). Despite their detection, however, these genes were not always significantly differentially expressed. The transcriptomic study of a population of *C. goreaui* from the central GBR is so far the first to show the upregulation of many genes by ≥ 4-fold in response to 13 days of exposure to elevated temperature (Levin et al. 2016). Differentially expressed genes included those belonging to ROS scavenging and protein-folding groups. Gene regulation in the Symbiodiniaceae under elevated temperature is understudied and thus very little is known about which genes or sets of genes may result in the differing thermal sensitivities between populations and species of the Symbiodiniaceae.

Finally, other human-derived pressures that interact with elevated temperatures on the Great Barrier Reef may exacerbate microalgal stress and coral bleaching. This includes pollutants such as herbicide additions into the ocean through terrestrial weed control and antifouling paints. On the GBR, summer coincides with monsoonal rainfall (Lough 2007; Kroon et al. 2012; Lough et al. 2015) and thus herbicide additions to the reef coincide with elevated temperatures. Diuron is one of the most commonly used herbicides in the GBR catchment and is particularly concerning due to its persistence, high mobility and potency (Owen et al. 2003; van Dam et al. 2012; Mercurio et al. 2016). Diuron targets the thermo-sensitive photosystem II of the photosynthetic apparatus of plants and microalgae and is designed to cause mortality. Chronic exposure of corals to diuron has led to coral bleaching (Cantin, Negri, & Willis, 2007; Jones & Kerswell, 2003; Jones, Muller, Haynes, &

Schreiber, 2003), while its combination with elevated temperature has resulted in the chronic photoinhibition of coral photosymbionts (Negri, Flores, Röthig, & Uthicke, 2011; van Dam et al., 2012). The combination of elevated ocean temperature along with other relevant stressors is largely understudied and is an important consideration for the health and stability of the coral-algal symbiosis.

Assisted evolution and the manipulation of coral photosymbionts

Organisms can respond to environmental change, such as ocean warming, through acclimation, adaptation or migration. For corals, however, many live near their physiological limits thus it is unlikely that physiological plasticity will enable them to mount a response great enough to cope with rapid ocean warming. In addition, corals generally have long sexual generation times of 2-20 years and thus adaptation is unlikely to occur fast enough to ensure their persistence into the future (Hughes et al., 2003). Finally, migration is limiting for corals due to the sessile nature of the adult life stage and the absence of suitable substrate for dispersed larvae. For the coral symbionts that play a vital role in coral survival and thermal response, asexual rates of reproduction are comparatively faster, with population generation times of ~70 to ~100 days *in hospite* (Falkowski et al. 1993). Thus, their rate of evolutionary adaptation through selection on random, beneficial mutations that have the potential to occur at each asexual generation, provides some hope for corals under in a warming world. Indeed, observations of increased coral bleaching tolerance after extreme heating events in a limited number of Indo-Pacific Reefs (Berkelmans & van Oppen, 2006), suggests that acclimation or adaptation is possible, although it is unknown which member in the holobionts was responsible for the increased tolerance.

It has recently been suggested that the thermal resilience of coral symbionts could be augmented through assisted evolution. Assisted evolution involves the acceleration of naturally occurring evolutionary processes to enhance certain traits (van Oppen et al. 2015; van Oppen et al. 2017) and has been proposed as a set of direct interventions and aims that increase coral climate resilience. Direct intervention at the scale of the microalgal symbionts is one of several aspects proposed in the assisted evolution of corals. In symbiosis with coral the Symbiodiniaceae live in a distinctively different state compared to the free-living state where symbionts in symbiosis may have a reduced metabolism (Goiran et al. 1996), rates of photosynthesis and the amount of carbon fixed and released may be host-controlled (Stat et al. 2008) and growth rates may be limited by nutrients provisioned by the host (Falkowski et al. 1993). In contrast, many populations of the Symbiodiniaceae have the ability to exist in a free-living state outside of the host, and when cultured

in nutrient replete conditions can exhibit generation times of up to a week (as opposed to up to 100 days in symbiosis (Falkowski et al. 1993)). The ability to culture Symbiodiniaceae with shorter generation times combined with the ease of carefully controlling their environmental conditions in a laboratory setting, allows evolutionary experiments to be undertaken in an attempt to evolve populations of the Symbiodiniaceae for environmental resilience, without the hindrance of the coral host. This form of assisted evolution uses experimental evolution, a technique that has been used across many microbial taxa (Reusch and Boyd 2013).

Experimental evolution is the study of evolutionary processes occurring in experimental populations in response to the conditions imposed upon them (Kawecki et al. 2012). It has been used to address diverse questions such as how a population may adapt to a specific environment, evolutionary trade-offs and constraints, and population genetic dynamics. Studies have mainly involved model organisms such as the bacteria *Escherichia coli* (e.g. Sniegowski et al., 1997; Travisano and Lenski, 1996) and *Drosophila* spp. (e.g. Mackay, 1980; Whitlock et al., 2002). More recently, marine microalgae have been subjected to climate change relevant scenarios over many generations, with several published studies using elevated temperature as a selective agent (e.g. Huertas et al. 2011; Flores-Moya et al. 2012; Schlüter et al. 2014). Some of these microalgae have exhibited a positive adaptive response compared to their wild-type counterparts. For example, the coccolithophore *Emiliania huxleyi* exhibited a 16% increase in growth after 460 generations of exposure to elevated temperature and pCO_2 (Schlüter et al. 2014), the dinoflagellate *Procentrum triestnum* exhibited up to 50% increase in growth rate after extended exposure to elevated temperature and reduced pH conditions (Flores-Moya et al., 2012), the same species exhibited a 12% increase in growth rate after 400 generations under elevated temperature and nutrients (Flores-Moya et al. 2008), while a multi-species study showed directed evolution over 120 generations was required for positive growth among populations of six phytoplankton species (Huertas et al. 2011). The latter study included two species of the Symbiodiniaceae that required 55 – 70 generations of evolution at 30 °C to exhibit growth rates that were equal to those at the control temperature. Apart from these studies, evolutionary experiments have rarely focussed on non-model organisms and never as a tool to enhance climate change resilience. Based on the successful adaptation of marine phytoplankton to environmental selection, experimental evolution could be a feasible tool to enhance the resilience of the Symbiodiniaceae to elevated temperature, or other climate-associated stressors.

Thesis outline and aims

This thesis examines experimental evolution of coral photosymbionts as a tool to increase their own thermal performance and that of the coral holobiont following reintroduction of the lab-evolved algae. Furthermore, it examines the molecular mechanisms that underly thermal tolerance.

In **chapter** 2, I first investigate whether experimental evolution can be used to evolve a thermally tolerant strain of *Cladocopium goreaui,* a common GBR species of coral photosymbiont. Specifically, growth rate, various photophysiological traits and levels of extracellular reactive oxygen species were measured to compare the response of thermally selected cells and wild-type cells at elevated temperature. Secondly, I investigate the comparative growth and bleaching tolerance of three GBR species of acroporid corals harbouring the thermally selected or the wild-type *C. goreaui* at both control and elevated temperature. These experiments allowed me to determine whether rapid adaptation using laboratory experimental evolution was possible for *C. goreaui* and what affect thermally selected cells had on holobiont bleaching thresholds.

In **chapter 3** I investigate the molecular pathways that underpin thermal tolerance in the experimentally evolved population of *C. goreaui.* To achieve this, I carried out a comparative transcriptomic study to examine gene expression differences in thermally selected compared to wild-type *C. goreaui* at elevated temperature. The results of this chapter highlight hallmark genes and functional pathways that could be targets for genetic engineering work in the future.

After investigating whether experimental evolution can be used as a successful approach to thermally evolve *C. goreaui,* **chapter 4** examines whether the same technique can be used to evolve populations of the Symbiodiniaceae across multiple species. Specifically, a 1 yr long thermal selection experiment using multiple temperature levels was followed by growth and photophysiological comparisons of selected and wild-type cells at elevated temperature. This allowed me to determine whether successful thermal adaptation across species of the Symbiodiniaceae had occurred and to investigate whether the maximum adaptive capacity of multiple Symbiodiniaceae species differed.

Finally, in **chapter 5** I first investigate the thermal and herbicide tolerances of the chromerids. Specifically, photophysiological traits of four chromerid populations were measured and compared with the Symbiodiniaceae species *C. goreaui.* Secondly the onset of symbiosis of each of the chromerid species was investigated across two species of coral in comparison to *C. goreaui*¸ under

both herbicide and elevated temperature conditions. This study allowed me to determine the tolerances of the chromerids to key stressors on the GBR as well as their ability to form a symbiosis with corals, as potential alternate or additional symbionts to the traditional Symbiodiniaceae.

Chapter 2: Rapid thermal adaptation in photosymbionts of reef-building corals

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2.1 Summary

Climate warming is occurring at a rate not experienced by life on Earth for 10s of millions of years and it is unknown whether the coral-dinoflagellate (family Symbiodiniaceae) symbiosis can evolve fast enough to ensure coral reef persistence. Coral thermal tolerance is partly dependent on the Symbiodiniaceae hosted. Therefore, directed laboratory evolution in the Symbiodiniaceae has been proposed as a strategy to enhance coral holobiont thermal tolerance. Using a reciprocal transplant design, I show that the upper temperature tolerance and temperature tolerance range of *Cladocopium goreaui* (formerly known as *Symbiodinium* ITS2 type C1) increased after ~80 asexual generations (2.5 years) of laboratory thermal selection. Relative to wild-type cells, selected cells showed superior photo-physiological performance and growth rate at 31°C *in vitro* and performed no worse at 27°C; they also had lower levels of extracellular reactive oxygen species (exROS). In contrast, wild-type cells were unable to photosynthesise or grow at 31°C and produced up to 17 times more exROS. In symbiosis, the increased thermal tolerance acquired *ex hospite* was less apparent. In recruits of two of three species tested, those harbouring selected cells showed no difference in growth between the 27°C and 31°C treatments, and a trend of positive growth at both temperatures. Recruits that were inoculated with wild-type cells, however, showed a significant difference in growth rates between the 27°C and 31°C treatments, with a negative growth trend at 31°C. There were no significant differences in the rate and severity of bleaching in coral recruits harbouring wild-type or selected cells. My findings highlight the need for additional Symbiodiniaceae species to be tested with this assisted evolution approach. Deciphering the genetic basis of enhanced thermal tolerance in the Symbiodiniaceae and the cause behind its limited transference to the coral holobiont in this species *C. goreaui* are important next-steps for developing methods that aim to increase coral bleaching tolerance.

2.2 Introduction

Climate warming is occurring at an unprecedented rate as a result of increasing atmospheric CO_2 and other greenhouse gasses (Hönisch et al. 2012; Zeebe et al. 2014) and has negatively affected terrestrial and marine ecosystems (Parmesan 2006). On coral reefs, rising seawater temperatures cause the breakdown of the critical association between the coral animal and its intracellular photosymbionts, belonging to the family Symbiodiniaceae, leading to bleaching and often coral mortality. Extensive bleaching-related loss of coral cover has occurred over the past 3-4 decades with the worst mass bleaching event on record having occurred most recently during the 2015-16 El Niño temperature anomaly (Eakin et al., 2016; Hughes et al., 2017; Normile, 2016) where over 60 % of reefs surveyed on the Great Barrier Reef (GBR) experienced extreme bleaching (Hughes et al., 2017). The frequency and severity of such heating events are anticipated to increase in the future with climate models predicting up to 99% of the world's coral reefs to experience severe annual bleaching before 2100 (van Hooidonk et al. 2016)

Increases in temperature can affect the Symbiodiniaceae and the coral host in a number of ways. The photosynthetic machinery of the Symbiodiniaceae is sensitive to even moderate temperature increases, causing photoinhibition and photodamage (Warner, Fitt, & Schmidt, 1999) of photosystem II (PSII, Iglesias-Prieto et al. 1992) and a disruption of the structural integrity of the chloroplast thylakoid membranes (Tchernov et al. 2004). Such changes limit the fixation of CO_2 (Murata et al. 2007), thus affecting Symbiodiniaceae cell growth. The transfer of photosynthate from the symbionts to the coral tissues accounts for up to 95 % of the coral's total energy requirements (Bythell 1988; Falkowski et al. 1993; Yellowlees et al. 2008) and a reduction in the amount of photosynthetically fixed carbon produced and translocated from symbiont to host can lead to reduced coral growth (Cantin, van Oppen, Willis, Mieog, & Negri, 2009; Goreau & Macfarlane, 1990) and tissue loss (Szmant and Gassman 1990). Furthermore, under heat stress, an excess production of reactive oxygen species (ROS) in the chloroplast can cause damage to DNA, proteins and lipids and in turn ROS can cause further detriment to photosynthetic activity by inhibiting PSII repair mechanisms (Murata et al. 2007) and damaging photosynthetic membranes (Lesser, 2006). Importantly, leakage of ROS out of the cell (extracellular ROS or exROS) by the Symbiodiniaceae, when produced in excess, has been linked to coral bleaching by stimulating the expulsion or digestion of the Symbiodiniaceae by host cells, or the removal of the Symbiodiniaceae by apoptotic host cell death (Weis 2008).

Many coral species live near their upper thermal limits and it is unlikely that physiological plasticity will enable them to mount a response great enough to cope with further environmental change (Hoegh-Guldberg et al. 2008). When physiological limits are exceeded, survival may depend on genetic adaptation through natural selection (Hoffmann and Sgrò 2011). However, the generally long sexual generation times of corals (2-20 years) in the face of rapid ocean warming, renders coral species unlikely to undergo significant adaptation in time to persist into the future (Hughes et al., 2003). Coral thermal tolerance is partly dependent on the genetic variant(s) of the Symbiodiniaceae that they host (Baker, 2003; Baker et al. 2004; Berkelmans & van Oppen, 2006; Howells et al. 2012; Mieog et al. 2009; Sampayo et al. 2008). A fast rate of asexual reproduction (three to 74 days [Wilkerson *et al.,* 1988]) for the Symbiodiniaceae, in combination with their large population sizes (~10^{10} cells in a branching coral ~30 cm diameter in size [van Oppen *et al.*, 2011]), has the potential to give rise to many spontaneous, random mutations over a short period of time, a small number of which may be beneficial to the changed conditions experienced due to climate change. These properties suggest a high adaptive potential exists in within the Symbiodiniaceae. However *in hospite*, host factors may actively retard the rate of asexual division in the Symbiodiniaceae where the host can regulate growth through carbon allocation (Stat et al. 2008) and the active digestion or removal of microalgal cells (Titlyanov et al. 1996). Such factors could slow Symbiodiniaceae generation times and thus rates of evolution *in hospite*. This has led to the proposal of *in vitro* laboratory evolution in the Symbiodiniaceae with subsequent inoculation of the coral host, to be used as a potential conservation or restoration tool in assisting corals in coping with ongoing ocean warming (van Oppen et al. 2015, 2017).

Despite the potentially high adaptive capacity of the Symbiodiniaceae, most warming perturbation studies on these coral photosymbionts have been carried out over short time-scales with treatment exposure lasting few generations. Although such experiments show that thermal acclimation, equating to temperature changes on a seasonal scale, are possible in the Symbiodiniaceae (Takahashi et al. 2013), little is known about the longer-term, evolutionary consequences of ongoing ocean warming. Only one study has examined the longer-term response of Symbiodiniaceae to ocean warming, reporting that strains CCMP 2429 and CCMP 2433 (isolated from *Heliofungia actiformis* and *Pocillopora damicornis*, respectively) from the GBR were able to grow at 30°C after 55-70 generations of temperature selection, where previously they could not (Huertas *et al.*, 2011). Long-term, evolutionary experiments involving other microalgae have mainly looked at their response to elevated pCO_2 and not temperature (Collins & Bell, 2004, 2006; Flores-Moya et al., 2012; Jin, Gao, & Beardall, 2013; Lohbeck, Riebesell, & Reusch, 2012) ;but see Flores-Moya *et al.*, 2012;

Schlüter *et al.*, 2014). Some of these experiments have provided evidence for the evolution of key fitness traits across multiple generations in the laboratory (Lohbeck et al. 2012; Flores-Moya et al. 2012; Jin et al. 2013), most commonly using reciprocal transplant experiments (Lohbeck et al. 2012; Jin et al. 2013; Schlüter et al. 2014) to infer adaptation.

To examine whether rapid thermal adaptation of the Symbiodiniaceae is possible, I conducted a laboratory selection experiment using a monoclonal culture of *Cladocopium goreaui* that is a common species associated with reef-building corals and widespread on coral reefs around the world (Franklin et al. 2012; Fabina et al. 2012; Thornhill et al. 2014). In this study, *C. goreaui* was isolated from a colony of *Acropora tenuis* on the Great Barrier Reef (GBR) and 16 culture replicates, each representing an independent evolutionary unit accumulating independent mutations, underwent a ratchet experiment (Huertas *et al.*, 2011) designed to select for increased temperature tolerance. After the ratchet experiment (lasting ~5 generations), the selected *C. goreaui* cells were cultured for a further ~73 generations (approximately 2.5 years) at 31°C, while the ancestral monoclonal culture from which it was derived (the wild type [WT]) remained at 27°C for the same period of time (Table 2.1). To determine whether the selected Symbiodiniaceae (SS) had adapted rather than (reversibly) acclimated to 31°C, replicate cultures of the SS and WT were subjected to a reciprocal transplant experiment (i.e., WT@27, SS@27, WT@31, and SS@31); the most direct approach for conferring adaptive change (Lohbeck et al. 2012; Jin et al. 2013; Schlüter et al. 2014; Merilä and Hendry 2014).

The mean fitness of the WT and SS was assessed based on a number of key traits. Six photo-physiological traits were measured; the photochemical efficiency of PSII in the dark (Fv/Fm) and the light (ΔFv/Fm'; indicators of the maximum and effective quantum yield of PSII, respectively), the maximum excitation pressure over PSII (Q_m, Iglesias-Prieto *et al.*, [2004]), the maximum electron transport rate (rETRm; an indicator of maximum photosynthetic capacity, Schreiber [2004]), the minimum saturating irradiance of photosynthesis (E_k, Henley, [1993]) and maximum non-photochemical quenching (NPQ_m; a photo-protective mechanism involving the dissipation of excess light energy as heat, Ralph *et al.*, [2002]). Two additional traits, the specific growth rate and the amount of exROS *per* cell were also compared between WT and SS cells at ambient and elevated temperature.

To understand the impact of *in vitro* thermal selection of *C. goreaui* on coral bleaching susceptibility, I inoculated aposymbiotic recruits of three GBR coral species, *Acropora cytherea, A. hyacinthus* and

A. sarmentosa, with the WT and SS cells and subjected them to heat stress. For this experiment, I used the same environmental conditions that were used in the *in vitro* reciprocal transplant experiment. After inoculation with the WT and SS, recruits were reared for five weeks at 27°C before being subjected to the different treatments for 28 days, during which their thermal tolerance was assessed based on growth (increase in recruit size) and the extent of bleaching (proportion of recruit surface that was pigmented).

My study tests the evolutionary consequences of culturing a species of the Symbiodiniaceae under thermal stress, adding to the important field of micro-algal adaptation through experimental evolution. Furthermore, my results provide insights into the use of experimentally evolved coral photosymbionts as a potential conservation or restoration tool that aims to increase coral thermal tolerance.

2.3. Materials and methods

***C. goreaui* isolation**

Cladocopium goreaui cells were extracted from the coral *A.* tenuis, Nelly Bay, Magnetic Island, Australia (19° 10’ 6’’ S, 146° 50’ 60’’E) in 2010. Extraction occurred by air-brushing the coral tissue from the coral skeleton, centrifuging (5 min, 1600 g), decanting, and re-suspending the pellet three times in 0.2 µm filtered seawater. Extracted cells were transferred into sterile culture media, Daigo’s IMK for Marine Microalgae (Nihon Pharmaceutical Co., Ltd) containing antibiotics (penicillin, neomycin, streptomycin, nystatin, final concentration [100 µg/mL] each, amphotericin final [2.5 µg/mL], plus GeO_2 final [50 µM] (Beltran et al. 2012). Cells were inoculated into fresh IMK+antibiotics monthly, for five months to minimise bacterial contamination. This heterogeneous culture was previously used by Howells et al. (2012) and Levin et al. (2016). Subsequently, cells were plated onto IMK+antibiotics and 1% agar. A monoclonal culture was created by picking cells from a single colony forming unit. Monoclonal cultures were grown in IMK without antibiotics. The monoclonal strain was confirmed as belonging to the subclade C1 based on the ITS2 rDNA region (GenBank accession number AB778664.1) and named SCF055-1. Annual sequencing checks confirmed that the culture did not become contaminated during longer-term laboratory culture.

***C. goreaui* thermal selection and long-term culture**

The monoclonal culture was maintained at 27°C and 65 ± 10 µmol photons.m^{-2}.s^{-1} (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle for ~6 months before undergoing a thermal selection experiment. Thermal selection was performed using a ratchet-design (Huertas et

al. 2011) lasting two months (~5 generations). The ratchet design is a method that maintains large population sizes by increasing the temperature in a step-wise fashion. With this approach, a population is only subjected to increased temperature (i.e., the next ratchet) when it shows positive growth. By maintaining population growth, this method maximises the number of spontaneous mutations arising from asexual cell division. With increasing levels of thermal selection pressure; natural selection can then act upon beneficial new mutations. This design allows the selection of best performers (assumed to be mutants as genetically homogenous monoclonal cultures are used) at each temperature ratchet.

Replicates (n = 16) of the monoclonal culture were inoculated into fresh media at 300 000 cells mL^{-1} and placed at 26°C. High replication was used initially to increase the chances of a spontaneous, beneficial new mutation occurring in at least one population. Importantly, each 'replicate' population was no longer considered a replicate once the experiment commenced, but an independent population representing a different random chance for a beneficial mutation to arise (Huertas *et al.*, 2011). After 30 days, the four populations displaying the highest growth at 28°C, or the greatest cell densities, were transferred to the next ratchet temperature (2°C higher than the previous) and split between four more vessels containing fresh media, at the same starting cell density, resulting in n = 16 populations at both 28°C and 30°C, respectively. Those kept at the next ratchet temperature of 32°C, however, did not exhibit any net growth and 30°C was considered their maximum adaptive capacity. The cells able to survive and grow at an elevated temperature are hereafter named the selected Symbiodiniaceae (SS) and their counterpart cells, that remained in control temperatures, named the wild-type (WT). Following the ratchet experiment, ten randomly selected WT populations were kept at 26.63 ± 0.001°C and ten of the fastest growing SS populations, resulting from the selection experiment, were kept at 31.16 ± 0.002°C and cultured separately for approximately 30 months (*ca.* 142 and 73 generations, respectively, see below for description on estimation of generation number). The ten WT populations were named SCF055-1-1 to SCF055-1-10 and ten SS populations were named SCF055-1-11 to SCF055-1-20. Temperature measurements were recorded every 10 min, using a data logger (HOBO Pendant® **Table 2.1**). Monthly, each WT and SS population was sub-cultured into fresh culture media (IMK).

Table 2.1. *In vitro* culture temperature conditions for the wild-type (WT) and selected Symbiodiniaceae (SS) cells. Pre-experimental temperature refers to the long-term culture history (210 weeks) of the WT and SS cells in control and elevated temperatures, respectively. Experimental phase temperatures are those experienced during the reciprocal transplant experiment (five weeks) by both the WT and SS cells during two weeks of pre-

acclimation and three weeks of experiment. Values are mean ± SEM. Number of replicate measurements are provided in parentheses.

Culture	Treatment	Temperature (°C)
Long-term	WT@27	26.63 ± 0.001 (76599)
	SS@31	31.15 ± 0.002 (77730)
Experimental	WT@27	26.85 ± 0.005 (5032)
	SS@27	
	SS@31	31.05 ± 0.002 (5032)
	WT@31	

Experiment 1: *In vitro* reciprocal transplant

Experimental design

I chose the SS population that had the greatest cell density at 31°C (SCF055-1-18), two weeks after sub-culturing, and a randomly chosen WT population (SCF055-1-3). To test whether adaptation in the SS cells had arisen from long-term acclimation or from genetic selection on beneficial mutations arisen through cell division the SS and WT cells then underwent a reciprocal transplant experiment. SS and WT cells were pre-acclimated for two weeks in their own (WT@27 and SS@31) or transplanted into reciprocal (WT@31 and SS@27) temperature conditions at a starting cell density of 200 000 cells mL^{-1}. The pre-acclimated cells were then transferred to culture vessels (25 cm^2, Corning®, Sigma-Aldrich) containing 10 mL of fresh media at a density of 200 000 cells mL^{-1} to give 12 replicate cultures for each of the four treatments. This replicate number was chosen so that at least three replicates could be used for the quantification of different physiological traits (see below).

During a previous study, using the heterogeneous population from which my WT strain was derived, heating for 15 days at 32°C revealed no difference in performance traits under elevated compared to ambient temperature conditions (Levin et al. 2016). Therefore, I did not carry out performance measurements until day 3, post-acclimation (representing 17 days in temperature treatment conditions). On days 3, 7, 10, 14, 17 and 21 (D3-D21) post-acclimation, measurements for photosynthetic performance and growth were taken in six replicates (replicates seven to 12). On D7, D14 and D21 post-inoculation, three replicates from each transplant (D7 -replicates one to three, D14 - four to six and D21 - seven to nine) were sacrificed for the measurement of extracellular ROS (exROS) production. Cells therefore spent a total of five weeks in experimental conditions, corresponding to up to eight asexual generations. For bacteria, seven generations are enough to attribute whether adaptation has occurred through genetic mechanisms or through acclimation

(Cooper 1991), where genetic adaptation is shown by the difference in growth rate of selected cells under the selective conditions (SS@31) with the growth rate of the wild-type cells that have been transferred to the selective conditions (WT@31).

Photosynthetic performance

The maximum quantum yield of PSII fluorescence *($F_v/F_m = F_m – (F_0/F_m)$)* was measured one hour before the end of the dark cycle, while the effective quantum yield ($\Delta F/F_m'$) of PSII was measured after six hours of light exposure. Both maximum and effective quantum yield measurements were carried out using an imaging pulse amplitude fluorometer (iPAM, Walz, Germany), with a Measuring Intensity of four, Saturating Intensity of seven, and Gain and Damping of two. PAM settings were chosen based on preliminary experiments as a compromise between fluorescence detection of low cell densities and signal saturation for high cell densities. The maximum excitation pressure over PSII (Q_m) was calculated using measurements for the maximum and effective quantum yields of PSII (Iglesias-Prieto *et al.*, 2004) using the equation

$$Q_m = 1 - [(\Delta F/F_m')/(F_v/F_m)]$$

Rapid light curves (RLCs) were carried out following $\Delta F/F_m'$ measurements by exposing each replicate culture to 10 steps of increasing actinic light (0-461 µmol photons m^{-2} s^{-1} PAR) each lasting 10 s. The RLCs for each replicate were fitted to the model by Platt et al. (Platt et al. 1980). The parameters of $rETR_m$ and E_k were calculated using SigmaPlot (Hill et al. 2004). By D17 and D21, the WT@31 replicates were no longer able to respond to the increases in light induced by the RLCs and data could not be fitted to the model. Values for E_k could therefore not be calculated at these time points. Non-photochemical quenching (NPQ) was calculated for each step of the RLCs using the equation

$$NPQ = \frac{F_m - F_m'}{F_m}$$

Values for the maximum NPQ (NPQ_m) were extracted from the highest PAR exposure during the RLCs.

Growth rate and estimation of cell generation number

For cell density measurements, aliquots of 50 µL from replicates six to 12 (n = 6) were fixed by adding 4 µL of 25% glutaraldehyde and stored at 4°C for later measurements of cell density. Cell density was determined by triplicate haemocytometer counts and specific growth rate (µ, doubling day^{-1}) was calculated as

$$\mu = \frac{ln\ N_1 - ln\ N_0}{\Delta t}$$

where N_0 is the cell density at D3, N_1 is the cell density at D17 and t is the duration (17-3) of culture in days. Cell density values for D3 and D17 were chosen as they represented the fastest growth rates during the experiment.

To estimate the number of generations that both the WT@27 had SS@31 had been through, both during their long-term culture and the reciprocal transplant experiment, I used cell density values from the three week experimental phase of the reciprocal transplant experiment. First, the doubling time (or generation time) was calculated according to the equation

$$T = 21\ x\ (\frac{\log(2)}{\log\left(\frac{N_1}{N_0}\right)})$$

where N_1 is the end cell density after 21 days of growth and N_0 is the cell density at the start of the experiment (200 000 cells mL^{-1}). Next the number of generations was calculated as

$$Generations = N/T$$

Where N is the number of days of long-term culture (e.g., 840 days post-ratchet experiment) and T is the doubling time. Although doubling times could have varied during the long-term culture, to provide an estimate of the generation number, I assume that they were constant.

Extracellular ROS production

For extracellular reactive oxygen species (exROS) determination, 1 mL of culture from each sacrificial replicate (n = 3) was pelleted (295 g/2000 rpm, 5 min) and 250 µL of the supernatant from each replicate was placed three times into a black, clear-bottom 96-well culture plate (Costar, Corning®, Sigma-Aldrich). The supernatant was incubated with 0.5 µL of CellROX Orange (Levin et al. 2016) (final concentration 5 µm, 25 min at 37°C), a fluorogenic probe that exhibits orange fluorescence upon oxidation by ROS (545 nm absorption, 565 nm emission). Fluorescence measurements were taken using a microplate reader (Synergy™ H4 Hybrid, Biotek®**)** immediately after incubation. Prior to pelleting, an aliquot of 50 µL from each sacrificial replicate was taken for the determination of cell density. The three fluorescence values for each replicate were then standardised to cell number to give relative exROS production *per* cell in arbitrary fluorescent units.

Experiment 2: *in hospite* reciprocal transplant experiment

Coral settlement and inoculation with C. goreaui

To determine whether thermal selection in *C. goreaui* could enhance the thermal tolerance of the holobiont I chose three species of coral, *Acropora cytherea, A. hyacinthus* and *A. sarmentosa.* Coral colonies of each species were collected from Trunk Reef, Great Barrier Reef, Australia (18° 18’ 101’’

S, 146° 52’ 226’’ E) and kept in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (Townsville, Australia) for three days before the full moon. Following spawning, gametes from four colonies of *A. cytherea* and five of *A. hyacinthus* and *A. sarmentosa* were each mixed in equal quantities (sperm concentration: ~10^6 mL^{-1}), within a species, for fertilisation. Resulting larvae were kept in aerated 0.4µm filtered seawater for *ca.* three weeks.

Six-well, multi-well plates (well volume 16.8 mL, Corning®, Sigma-Aldrich®) were prepared with a settlement cue- the peptide Hym-248 (Iwao et al. 2002), by placing three, 3 µL droplets of 60 µM in each well, and leaving to dry. These droplets were placed at equal distance from the edge of each well using a template placed underneath the plate. Planulae were taken from the SeaSim and washed three times in 0.2 micron filtered seawater (FSW). Three, 90 µL droplets of FSW, each containing one planula, were plated into each well on top of the dried peptide dots. Planulae were left for 12 h in their droplets, in the dark, at 27°C to metamorphose and settle. Subsequently, each well was filled with 9 mL of FSW and planulae that had not metamorphosed or settled were removed. This method resulted in 134 individual recruits of *A. cytherea* across 14 plates, 136 of *A. hyacinthus* across 14 plates and 125 of *A. sarmentosa* across 15 plates. Coral recruits were reared at 27 °C, 65 ± 10 µmol photons.m^{-2}.s^{-1} under a 14:10 light:dark cycle and were supplemented twice weekly with a 0.2 micron, filter-sterilised cocktail of nutrients, glycerol, vitamins and amino acid as in Wang et al. (2012). Plates were placed on a slow-moving, rotating shaker plate to allow the homogenisation of food. Water changes were carried out the day after feeding by removing the water in each well and replacing to total of 9 mL *per* well with fresh FSW.

Experimental design

Nine days post-settlement, half of the plates containing recruits were inoculated with WT cells and half with SS cells (pre-acclimated to 27°C for two weeks to 30 000 cells mL^{-1}) and left for two days before replacing each well with fresh FSW. Inoculation was repeated 12 days post-settlement to maximise the number of symbionts taken up by the recruits. Five days after the initial inoculation, recruits had visually taken up symbionts and they were subsequently reared for 5 weeks at 27°C to ensure the symbiosis was stable and recruits were sufficiently pigmented for further analyses. Subsequently, half of the plates containing corals infected with the WT and half of those containing corals infected with the SS were transplanted into 31°C (D0) while the remaining recruits were kept at 27°C. This resulted in four experimental treatments, which mirrored those of the *in vitro* experiment (*i.e.*, WT@27, SS@27, SS@31, and WT@31). For each coral species, this resulted in three to five replicate plates and between 27 and 38 individual recruits *per* treatment. Replicate number

was considered sufficient to take into account potential variation in recruit physiology as a result of genetic variation.

Recruit size and extent of pigmentation

Images of each recruit were taken using a camera-stereomicroscope setup (SMZ800N, Nikon) on the day of transplantation (D0), two weeks (D14) and 4 weeks (D28) post-transplantation. Images were taken while the polyp's tentacles were retracted. The same camera settings were globally applied to each image. Recruit size was determined as the base area of each recruit using imaging software (NIS-Elements BR v430, Nikon). This was done by detecting the outline of each recruit base and auto-calculating the area within (region of interest (ROI), **Figure 2.1a**). Growth was expressed as the percentage change in size of each recruit at D28 from the starting size at D0.

As a proxy for bleaching, I tracked any changes in percentage pigmentation of the surface area of each recruit during the experiment. To do this, I used the images from D0, D4 and D8 with the recruit set as the ROI. The pigmented areas within the ROI were detected by setting red, green, blue (RGB) threshold limits for each colour channel (Red: 90-155, Green: 70-100, Blue: 0-105). The same RGB threshold limits were applied across all images and values of area pigmentation were expressed as a percentage of the total area of each recruit (**Figure 2.1b**).

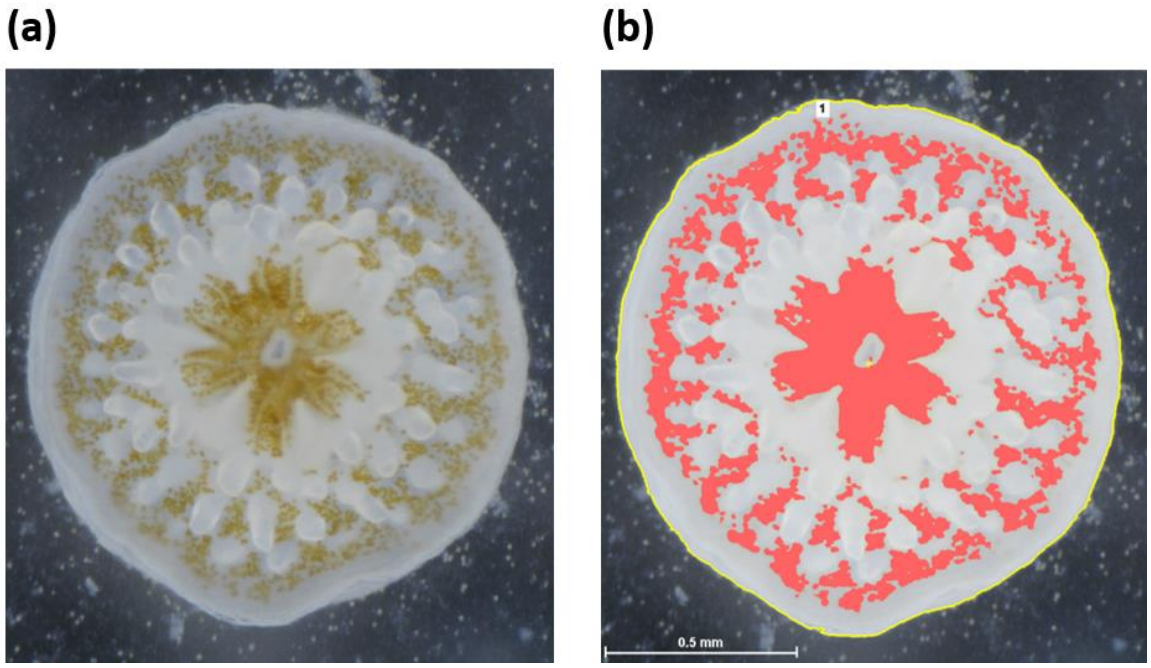

Figure 2.1 Determination of recruit base area and area pigmentation (%) of a coral recruit. (**a**) Raw image of a coral recruit, (**b**) same recruit image with the region of interest (ROI) selected (recruit base area) and the area pigmented highlighted after applying red, green blue (RGB) thresholds. The area pigmented is expressed as a percentage of the total base area of the recruit.

Experimental water parameters

Throughout both the *in vitro* and *in hospite* experiments, temperature conditions were measured every 10 min with a HOBO data logger (Tables 2.1 and 2.2). Light measurements were checked daily during the middle of the light cycle and were always 65 ± 10 µmol photons.m^{-2}.s^{-1}. For the *in hospite* experiment, pH (NBS scale specific to seawater), salinity and oxygen saturation measurements were taken from one random well *per* plate, twice weekly, coinciding with water changes (**Table 2.2**) to make sure a high water quality was maintained.

Table 2.2. *In hospite* rearing conditions for coral recruits colonised with either the wild-type (WT) or selected Symbiodiniaceae (SS) cells in two temperature conditions. Pre-experimental conditions refer to the ambient temperature rearing conditions of recruits infected with both the WT and SS cells (five weeks) before half being moved into elevated temperature treatment during the experimental phase (three weeks). Values are mean ± SE. Number of replicates are provided in parentheses.

Phase	Treatment	Temperature (°C)	Salinity (˚/$_{00}$)	Dissolved Oxygen (%)	pH_{NBS}
Pre-experimental	WT@27	26.81 ± 0.005 (5183)	33.13 ± 0.35 (127)	97.89 ± 0.12 (127)	8.06 ± 0.005 (127)
	SS@27		33.6 ± 0.06 (138)	97.87 ± 0.10 (138)	8.06 ± 0.005 (138)
Experimental	WT@27	26.81 ± 0.007 (2971)	33.36 ± 0.07 (66)	96.83 ± 0.18 (66)	8.09 ± 0.008 (66)
	SS@27		33.49 ± 0.09 (66)	96.12 ± 0.53 (66)	8.10 ± 0.008 (66)
	SS@31	31.09 ± 0.002 (3075)	33.53 ± 0.09 (66)	95.59 ± 1.15 (66)	8.10 ± 0.007 (66)
	WT@31		33.53 ± 0.09 (78)	95.92 ± 0.02 (78)	8.10 ± 0.007 (78)

Statistical analyses

In vitro: To analyse the effects of temperature on the WT and SS cells, I compared the physiological responses of WT and SS cells in their own (WT@27, ST@31) and reciprocal (ST@27 and WT@31) temperature treatments across time, where relevant. To test the effects of treatment on *in vitro* specific growth rate and photo-physiological traits, I used a linear model with 'treatment' (i.e. WT@27, ST@31, ST@27 and WT@31) as a fixed factor and with 'time' as an additional fixed factor for all photo-physiological traits. The effect of treatment and time on exROS production was tested using a generalized linear mixed effects model fitted for a poisson distribution, again with 'treatment' and 'time' as fixed factors.

In hospite: Similarly, to analyse the effects of temperature on the coral recruits infected with the WT and SS cells, I compared the physiological responses of corals infected with WT and SS cells in their own (WT@27, SS@31) and reciprocal (SS@27, WT@31) treatments across time, where relevant. Linear mixed effects models were used to test the effects of treatment and time on the % change in growth for each coral species, while generalized linear mixed effects models fitted for a negative binomial distribution were used to test the effects of treatment and time on area pigmentation (%) of recruits for each coral species. In both cases, 'treatment' and 'time' were fixed factors and 'plate' was included as a random fixed factor.

All analyses were performed in R (v. 3.3.2, R Core Team, (2016)), with annotated scripts and raw data included in the Supporting Information (S1 and S2, respectively). Linear models and linear mixed effects models were carried out using the package 'nlme'(Pinheiro et al. 2017), while generalized linear mixed effects models were carried out using the package 'lme4' (Bates et al. 2015). For both *in vitro* and *in hospite* traits, all pairwise comparisons were carried out *post-hoc* with Tukey's tests using the package 'multcomp'(Hothorn et al. 2008). Heterogeneity and normality were assessed using Residual and Q-Q- plots. Where 'time' was a fixed factor, I checked for correlation within the model and in all cases, none was detected.

2.4 Results

Heat stress responses of *C. goreaui in vitro*

Photosynthetic performance

Maximum and effective quantum yield values (Figs. 2a, b) for the SS@31 were not significantly different to those for the WT@27 and SS@27 throughout most of the experiment but were higher than mean values for the WT@31 (max. $p < 0.05$). The WT@31 values were always significantly lower (max. $p < 0.05$) compared to those in the remaining treatments, with the mean Fv/Fm significantly decreasing over time from 0.46 (±SEM, ±0.007) to 0.37 (±0.004) and mean ΔF/Fm' from 0.41 (±0.002) to 0.22 (±0.036) from day 3 (D3) to day 21 (D21, $p < 0.05$). The mean maximum excitation pressure over PSII (Q_m, **Figure 2.2c**) for the WT@31 was significantly elevated over five of the six time points, compared to the other treatments (max. $p < 0.05$), and by D21 the mean Q_m had significantly increased to 0.42 (±0.054) from 0.11 (±0.017) at D0 ($p < 0.05$). The mean irradiance at onset of light saturation (E_k), maximum relative electron transport rate ($rETR_m$) and maximum NPQ (NPQ_m) for the WT@31 were significantly lower than for the remaining experimental groups (max. $p < 0.05$) across the duration of the experiment (Fig 2d-f) with the mean $rETR_m$ significantly decreasing

over time ($p < 0.05$). One exception was at D7, when the mean E_k value was not statistically different from that in the SS@27 and WT@27 treatments (min. $p > 0.05$).

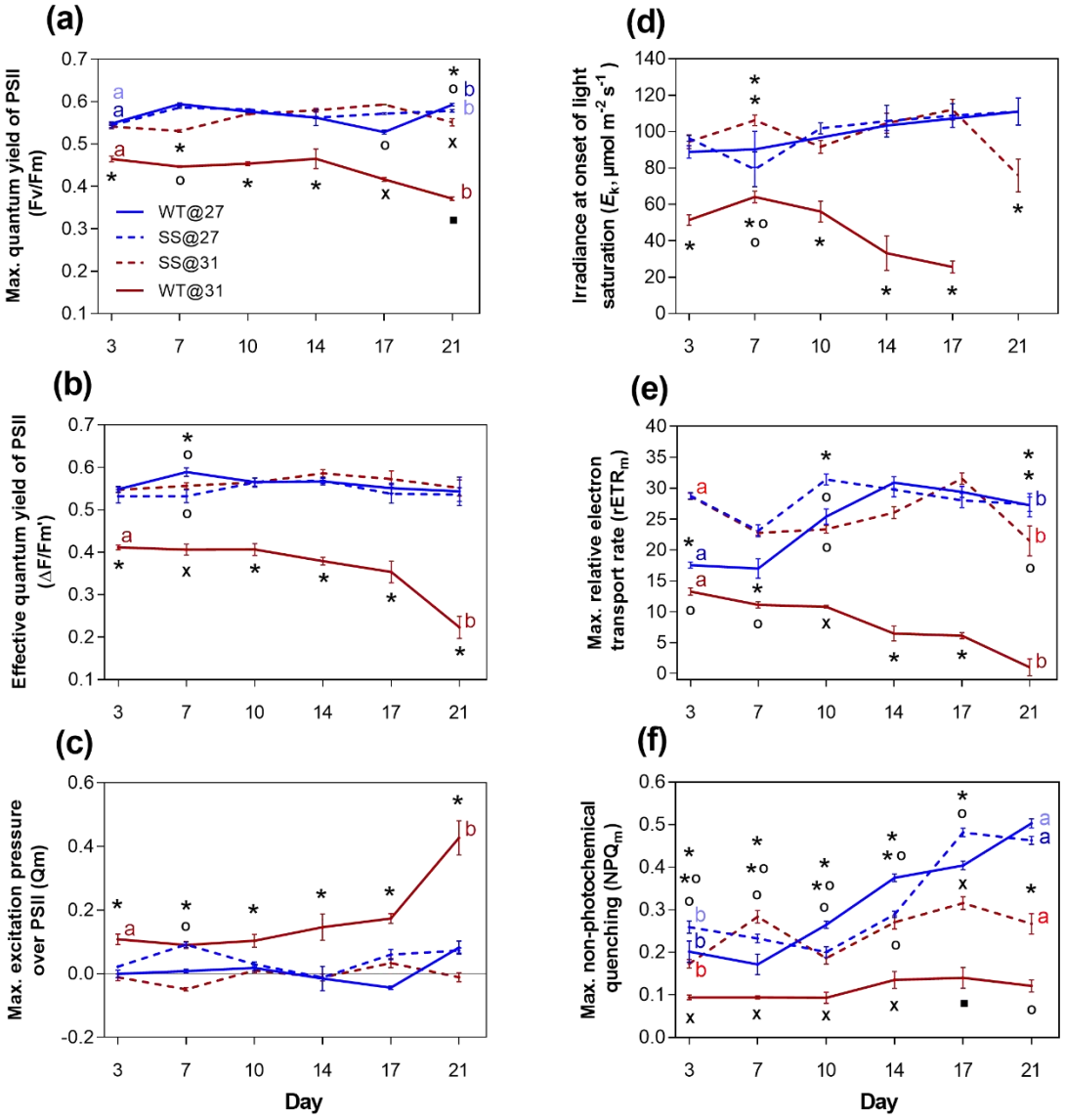

Figure 2.2 Comparison of mean (±SEM) photochemical performance traits in wild-type (WT) and selected (SS) *Cladocopium goreaui* at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31) over 21 days after 14 days of pre-acclimation. (a) Maximum quantum yield of photosystem II. (b) Effective quantum yield of photosystem II. (c) Maximum excitation pressure over photosystem II. (d), Irradiance at onset of light saturation. (e) Maximum relative electron transport rate. (f) Maximum nonphotochemical quenching. Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) within a time point are represented by different symbols above/below the line plots, while statistically significant differences over time (between D3 and D21) for each treatment are represented by different letters (Tukey's post hoc tests; p < .05). n = 6 for all means. Where error bars are not visible, they are small and hidden by the symbols

Specific growth rate

The mean growth rate of SS@31 was positive at 0.07 d^{-1} ±0.005. Although this was significantly slower than the growth rates of the WT@27 (0.16 ±0.007) and SS@27 (0.14 ±0.008, max. $p < 0.05$),

all three had significantly greater mean growth rates compared to the WT@31 (max. $p < 0.05$), which exhibited negative growth at -0.05 μ (±0.010, **Figure 2.3a**).

Oxidative stress: ExROS of the WT@31 had mean values that were always significantly greater than those observed for the other experimental groups (max. $p < 0.05$) and that significantly increased over time from 46.2 (±2.04) at D7 to 105.62 (±22.25) by D21 ($p < 0.05$, **Figure 2.3b**). By comparison, the highest mean value of SS@31 throughout the experiment was 22.19 (±4.10). The amount of ExROS produced in the SS@31 treatment was not significantly different to that of the SS@27 or WT@27 at any time point ($p > 0.05$) and the production of exROS by SS@31, SS@37 or SS@27 did not significantly change over time ($p > 0.05$).

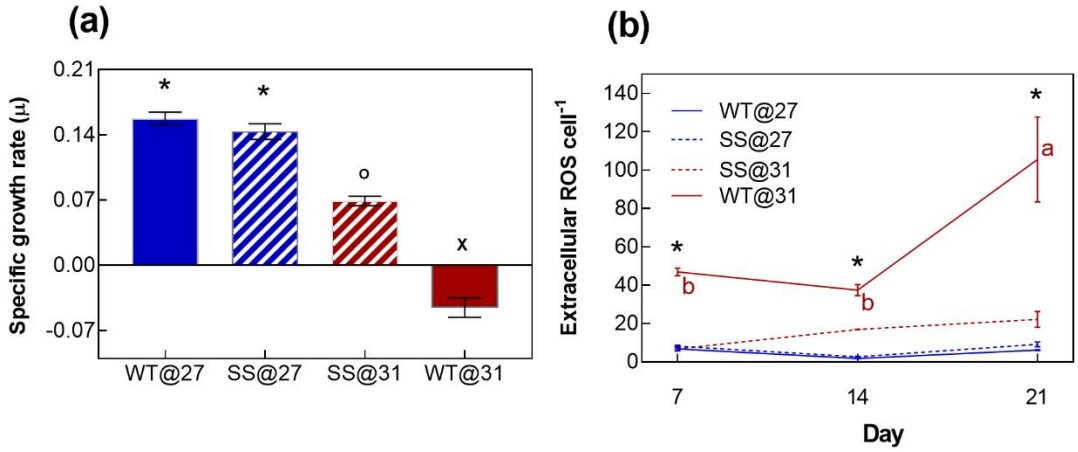

Figure 2.3 Comparison of mean (±SEM) growth and oxidative stress of wild-type (WT) and selected (SS) *Cladocopium goreaui* at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31) after 14 days of pre-acclimation. (a) Specific growth rate (n = 6) calculated from cell densities at D3 and D17. (b) Extracellular reactive oxygen species production per cell (n = 3). Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) are represented by different symbols above/below the bar/line plots (Tukey's post hoc tests; p < .05) (a, b). Statistically significant differences over time for each treatment are represented by different letters (b). Where error bars are not visible, they are small and hidden by the symbols

Heat stress responses of the juvenile coral holobiont

Growth

Measurements of percentage change in size over the 28-day experiment (Figs. 4a-c) revealed no significant differences in growth between the WT@27, SS@27 and SS@31 recruits for each coral species (min. $p = > 0.05$), which generally showed a trend of positive mean growth with increases up to 1.29 ±0.52% over the four-week experiment. In contrast, WT@31 recruits displayed a trend of negative mean growth for all three coral species. Despite this, the mean growth of the WT@31 recruits was not significantly different from that of the SS@31 recruits for all three coral species (min. $p > 0.05$), although it was significantly lower compared to that of the WT@27 recruits for *A.*

cytherea and *A. hyacinthus* (max. $p < 0.05$). The WT@31 mean growth was significantly lower compared to that of the SS@27 recruits for *A. hyacinthus* and *A. cytherea* ($p < 0.05$).

Area pigmented

There were no significant differences in mean percentage pigmentation at D0 for all coral species harbouring either the ST or WT cells ($p > 0.05$, Figs. 4d-f). By D14 the mean percentage pigmentation of all three coral species harbouring the SS and WT cells had not significantly changed in the 31°C treatment ($p > 0.05$), while the majority of those harbouring the WT and SS at 27°C had increased ($p < 0.05$). While the recruits harbouring WT cells were significantly less pigmented at 31°C than those at 27°C across the duration of the experiment (max. $p > 0.05$), the mean percentage pigmentation for recruits in the SS@31 treatment across all three species was not significantly different to the recruits harbouring the WT or ST cells at 27°C (min. $p > 0.05$). The only exception was *A. hyacinthus*, where the mean percentage pigmentation for SS-recruits at 31°C was significantly lower than the for WT-recruits at 27°C ($p < 0.05$, **Figure 2.4e**). By D28, WT and SS recruits of all three species had bleached at 31°C (0.75-6.51% mean pigmentation), with mean pigmentation values not significantly different between the WT and SS recruits (min. $p > 0.05$) as well as being significantly less pigmented compared to the recruits at D0 (min. $p > 0.05$).

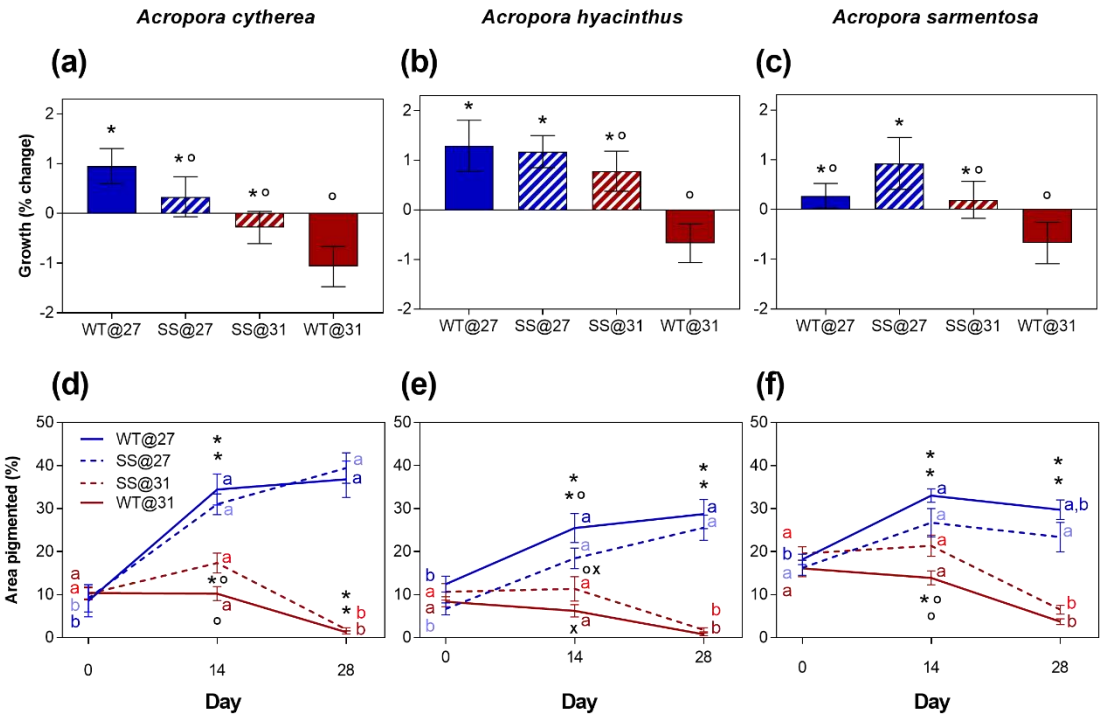

Figure 2.4 Comparison of mean (±SEM) growth and bleaching of coral recruits harbouring wild-type (WT) and selected (SS) *Cladocopium goreaui* at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31). (a–c) % Change in size between D0 and D28 for three coral species (n = 25–34). (d–f) Proportion of recruit surface area pigmented (%) for each coral species (n = 20–37). Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) within a time point are represented by different symbols above/below the

line plots (Tukey's post hoc tests; $p < .05$, a–f). Statistically significant differences over time (d–f) for each treatment are represented by different letters (Tukey's post hoc tests; $p < .05$). Where error bars are not visible, they are small and hidden by the symbols

2.5 Discussion

The stable phenotypic changes observed in the SS cells after ~80 generations (2.5 years) of *in vitro* selection provides compelling evidence of rapid thermal adaptation in *Cladocopium goreaui*. My study is one of only a few micro-algal studies that show thermal adaptation through experimental evolution. SS cells significantly outperformed the WT cells at elevated temperature for all traits measured while they maintained performance levels similar to those of the WT cells at ambient temperature, demonstrating that laboratory selection has widened rather than shifted the SS temperature tolerance range for this strain. This is an important characteristic considering the often large natural daily and seasonal temperature fluctuations experienced on coral reefs (Kline et al. 2015). Contrary to expectations, I found the positive effects of thermal adaptation of the SS cells to be considerably reduced *in hospite*, although a trend of positive growth was observed in two of three coral species tested under heat stress when inoculated with SS cells, but not with WT cells.

Thermal adaptation and broadening of thermal range in algal endosymbionts *ex hospite*

All photo-physiological traits measured showed a significantly lowered performance of the WT@31 compared to the other experimental groups over the 21 day study period. In contrast, the SS@31, could maintain photosynthetic function similar to the WT@27 and SS@27 throughout the experiment. The specific growth rate and exROS data confirm an increase in temperature tolerance for the SS relative to the WT cells. The mean growth rate of SS@31, while up to 44% slower than that of the WT@27 and SS@27, exhibited positive growth (0.07 doublings day^{-1}), while the WT@31 exhibited mean negative growth of -0.05 doublings day^{-1}. Elevated temperature inevitably requires increases in metabolic activity (Beardall and Raven 2004). Selection on more efficient metabolic pathways for the SS may have been enough to maintain energetically costly photosynthetic processes (and/or photosystem and cellular repair mechanisms) as well as positive growth at 31°C, but not enough to sustain growth rates to match those at 27°C. Reduced growth rates as a result of greater thermal tolerance have been described previously through both short-term acclimatory and adaptive responses; A thermally tolerant isolate of the *Symbiodinium* genus (formerly *Symbiodinium* ITS2 type A1) cultured for 10 days at 32°C exhibited only a slight decline in photosynthetic activity but a considerable decline in growth, compared to control temperature conditions (Robison & Warner, 2006). In another example, evolution of thermal tolerance to elevated temperature in the phytoplankton *Chlorella vulgaris* resulted in a down-regulation in respiration relative to photosynthesis through changes in energy allocation (Padfield et al. 2016).

ExROS production by the WT@31 had mean values that were always higher than those for the remaining experimental groups and up to 17 times greater by D21. Conversely, the SS@31 was able to maintain lower exROS levels similar to the WT@27 and SS@27 throughout most of the experiment. Reactive oxygen species are molecules produced as a by-product of photosynthesis and respiration, as well as due to cellular stress. If produced in excess and not neutralised by antioxidant molecules and enzymes, ROS are secreted extracellularly into their surrounding medium. Excessive ROS production *in hospite* has been linked to triggering the bleaching response of corals (Downs *et al.*, 2002; Lesser, 2006), thus their production is an important trait to consider in Symbiodiniaceae thermal selection experiments. Cellular ROS production can occur as a result of uncoupling of the photosystems (PSII and PSI) during heat stress (Iglesias-Prieto et al. 1992), and in excess can cause damage to DNA, proteins, lipids and cell membranes (Tchernov *et al.*, 2004; Lesser, 2006; Venn *et al.*, 2008). The high exROS produced by the WT@31 likely explains the inability for growth and severe photo-inhibition at an elevated temperature.

Two previous studies have experimented with the heterogeneous population from which my WT strain was derived. Howells *et al.* (2012) showed that, after 11 days of heating, *C. goreaui* from Magnetic Island showed no signs of stress at 32°C and displayed maximum quantum yields 16 % higher than at the control temperature of 27°C. In a more recent study, heating of the same *C. goreaui* for 15 days at 32°C revealed no difference in photosynthetic performance, or amount of exROS compared to culture replicates maintained under ambient temperature conditions. Heat-exposed cells were able to acclimate via upregulation of a number of antioxidant and molecular chaperone genes (Levin et al. 2016), preventing damage to the photosynthetic apparatus and allowing cell growth to be maintained. In my study, D3 already represented 17 days of exposure to 31 °C for the WT@31 as a result of two weeks pre-acclimation to experimental conditions. The reduced performance of my WT@31, and subsequent decline in fitness, indicates that more than 15 days are needed before thermal stress significantly impacts the WT's physiological performance, this informs times scales of experimentation that are appropriate for assessing divergence in performance. The energetic costs of upregulating pathways to combat the effects of thermal stress could have become too high for thermal tolerance to be maintained (DeWitt et al. 1998). Maintenance of low exROS levels by the SS@31 could be a result of a greater antioxidant capacity under heat stress (McGinty et al., 2012) compared to the WT, perhaps through the selection of cells with a higher metabolic capacity able to support such costly antioxidant responses. Alternatively, a

more stable photosynthetic apparatus, through alterations in the fatty acid composition of thylakoid membranes (Tchernov et al. 2004) for example, could limit cellular ROS production in the SS cells.

Climate change-relevant evolutionary experiments have been carried out on other microalgae, although none of these previously studied algae have a symbiotic life stage. These microalgal studies have mainly focussed on the effects of selection to an elevated pCO_2 regime, and often used growth rate as the only fitness parameter measured. Some have failed to show adaptation (Crawfurd et al. 2011; Tatters et al. 2013), while others have provided evidence for adaptation to elevated pCO_2 (Collins and Bell 2004; Lohbeck et al. 2012; Jin et al. 2013). A limited number of studies have investigated the evolutionary consequences of selection to elevated temperature; the dinoflagellate *Alexandrium minutum* adapted to a combination of elevated temperature and pCO_2 after ~250 generations (two years) of selection (Flores-Moya et al. 2008), a number of phytoplankton strains showed interspecific differences in thermal adaptive capacity (Huertas *et al.*, 2011) and the coccolithophore *Emiliania huxleyi* showed a 16 % higher growth rate in thermally adapted populations after 460 generations (one year) of selection (Schlüter et al. 2014). My results show that thermal adaptation is possible after only ~80 generations (2.5 years) of selection in *C. goreaui*. Such rapid adaptation may be in part be due to the nature of my initial experimental design (ratchet technique, [Huertas et al., 2011]), where sequential increases in temperature, only to levels that maintained population growth, meant maximising the potential for beneficial, random mutations to occur and thus maximising the rate of adaptation. Additionally, a further culture of ~73 generations (more than 2 years) at elevated temperature after the ratchet experiment could have allowed any such mutations to become fixed in the population.

Thermal adaptation of algal endosymbionts shows limited expression in symbiosis

While my *in vitro* results provide compelling evidence for an adaptive response to temperature selection in *C. goreaui*, the benefits of temperature adaptation showed limited expression in symbiosis with juvenile *Acropora* corals. There were no significant differences in tissue pigmentation between treatment groups and all were equally bleached at the end of the experiment when under heat stress. At the higher experimental temperature of 31°C there was no significant difference in growth rates between juveniles that harboured SS or WT cells but two of the three coral species harbouring the SS cells exhibited a trend of positive growth, while all species harbouring the WT displayed a trend of negative growth. Recruit growth at 27 °C was independent of infection with WT or SS cells, indicating no negative impact of thermal adaptation of the SS on holobiont growth at ambient temperature in this experiment. This contrasts with other Symbiodiniaceae studies that

have shown that higher thermal tolerance in *Durusdinium* (formerly ITS2 clade D) comes at the cost of reduced growth for *A. millepora* juvenile and adult corals at ambient temperatures, as well as *Pocillopora damicornis,* compared to corals harbouring more thermo-sensitive *C. goreaui* (Little et al. 2004) and another *Cladocopium species* formerly known as *Symbiodinium* ITS2 type C2 (Jones and Berkelmans 2010) and (Cunning et al. 2015a), respectively.

Differences between the *in vitro* and *in hospite* responses under heat stress have previously been reported for other Symbiodiniaceae strains (Bhagooli and Hidaka 2003; Goulet et al. 2005). My results, in conjunction with the observed differences in bleaching responses of different coral species that harbour the same Symbiodiniaceae species (Abrego, Ulstrup, Willis, & van Oppen, 2008; Ray Berkelmans & van Oppen, 2006b; Fisher, Malme, & Dove, 2012), confirm that the coral host contributes considerably to the bleaching tolerance level of the coral-Symbiodiniaceae holobiont (Baird et al. 2008). Little is known about potential host-factors influencing the holobiont response, but a number of species-specific variables have been suggested whereby the coral host can alter the algal microenvironment through differences in the amount and type of light reaching symbionts *in hospite*, host-based pigments (Dove 2004; Salih et al. 2006), host skeletal morphology (Enríquez et al. 2005; Kaniewska et al. 2008) and tissue thickness (Loya et al. 2001). Further, a potentially dissolved inorganic carbon (DIC) limited environment *in hospite* (Leggat *et al.*, 2000; Marubini *et al.*, 2008; Jarrold *et al.*, 2013) could drive differences in photosynthesis between *in vitro* cultures that may be DIC-replete. Alternatively, either the production of ROS by the host itself under heat stress, or host antioxidant capacity could have been so high that the increase in ROS produced by WT symbionts at 31°C was relatively insignificant. Furthermore, a breakdown of symbiont-host communication under stress has been suggested to affect the ability of a coral to discriminate between healthy and unhealthy Symbiodiniaceae cells (Baird et al. 2008), which could explain factors such as host-digestion of the Symbiodiniaceae, symbiont expulsion or apoptotic host cell death (Dunn et al. 2002; Weis 2008) as a resulting host response, regardless of Symbiodiniaceae health (Baird et al. 2008).

Assisted Symbiodiniaceae evolution to enhance coral bleaching tolerance

My results highlight the ability of a species of Symbiodiniaceae to evolve greater thermal tolerance over ecological time-scales relevant to the pace of climate change. A previous directed evolution experiment on the Symbiodiniaceae showed positive growth after 55-70 generations of culturing at 30°C , while no growth was observed at this temperature prior to selection (Huertas *et al.*, 2011).

These findings indicate that the ability for the rapid temperature adaptation is not limited to *C. goreaui* used in my study.

I show that the positive effect of laboratory selection on my particular *C. goreaui* strain was reduced in symbiosis. Many Symbiodiniaceae species are known to occur both free-living in coral reef sediments and the water column and in symbiosis with reef-building corals and/or other reef organisms Huang et al., 2013; Littman & Willis, 2008), such as the C1 type studied here. My findings suggest that fast growth may be an important trait to artificially select for when the goal is to enhance thermal tolerance in free-living Symbiodiniaceae, but may not be an appropriate trait to select for *in vitro* if the aim is to augment thermal tolerance *in hospite* (i.e., coral bleaching tolerance) (van Oppen et al., 2015). Additional laboratory selection experiments using several selection pressures and targeted traits are required to resolve this issue. Furthermore, the Symbiodiniaceae is a highly diverse family comprising many species (Baker 2003; Manning and Gates 2008; LaJeunesse et al. 2018) and it is possible that other Symbiodiniaceae species and strains within species may respond to laboratory thermal selection in different ways with varying effects on the holobiont. Finally, it is important to acknowledge the numerous other microbes (e.g. bacteria, fungi, archaea) that are present in corals. These microbes can have vastly different physiologies and can play important, functional roles (Ainsworth et al. 2010). Their interaction with the Symbiodiniaceae could therefore have implications in the overall phenotype of both the microalgae and the coral holobiont.

Models of reef futures under climate change have taken into account existing variation in symbiont thermal tolerance (Baskett et al. 2009) and coral resilience (Baskett et al. 2014). The results presented here on the rate of evolution in a species of the Symbiodiniaceae to rising seawater temperatures, and the potentially different selective forces in the free-living versus the symbiotic life stage (Day et al. 2008), are critical to refine such models. Examination of cellular pathways (e.g., *via* transcriptome analysis) underpinning the SS and WT Symbiodiniaceae thermal stress response *in vitro* and *in hospite* will likely provide insights into the distinct phenotypic responses observed here and may reveal why the positive effects of thermal selection were reduced *in hospite* in my experiment. Such studies will inform and improve experimental evolution studies using the Symbiodiniaceae for augmenting coral bleaching tolerance and their ultimate use in coral reef conservation and restoration initiatives.

Chapter 3: Gene regulation underpinning increased thermal tolerance following directed evolution in a coral photosymbiont

Leela J. Chakravarti, Patrick Buerger, Rachel A. Levin , Madeleine J. H. van Oppen

3.1 Summary

Coral reefs are threatened by unprecedented ocean warming. Even small increases in ocean temperature can disrupt the obligate symbiosis that corals form with dinoflagellate microalgae (family Symbiodiniaceae) resulting in coral bleaching. Bleached corals are vulnerable to starvation and three pan-global bleaching events have led to mass mortality and loss of coral cover. The upper thermal tolerance limit of the coral-algal symbiosis is partly dependent on the Symbiodiniaceae partner. While the physiological response of free-living Symbiodiniaceae to elevated temperature has been investigated, very little is known about the genes that underlie these responses. Here I compare the differential gene expression and functional pathways underpinning thermal tolerance in a wild-type (WT) culture of a Symbiodiniaceae species common on the Great Barrier Reef, *Cladocopium goreaui*, and an experimentally evolved culture (SS) derived from this WT. The SS culture underwent long-term (~80 generations) of laboratory thermal selection resulting in increased thermal tolerance compared to the WT, after which the transcriptomic responses of both cultures were compared at elevated and control temperatures. Thousands of genes were differentially expressed between the WT and SS cultures at a fold-change of >8 measured over a time-course of 35 days. At elevated temperature, WT cells exhibited a temporally unstable transcriptomic response with the majority of differentially expresed genes being upregulated. Upregulated genes included those involved in a classic stress response such as molecular chaperoning, protein repair, protein degradation, DNA repair and apoptosis, while photosynthesis genes were consistently downregulated across time points. In comparison, the thermally selected cells exhibited a more stable transcriptomic response over time, upregulating genes involved in transcription factor activity and genes homologous to the bacterial type two secretion pathway. Stress response genes that were upregulated for the WT cells, such as programmed cell death pathways and chaperonin activity, were downregulated in the SS cells. This study identifies key genetic pathways that underly thermal tolerance in a heat-selected, thermally tolerant population of *C. goreaui.* Such genes and functional pathways could be used to inform future genetic manipulation

experiments designed to develop thermally resilient strains of Symbiodiniaceae for use in coral restoration and conservation initiatives.

3.2 Introduction

Tropical coral reefs are increasingly under threat from rapid global warming caused by the anthropogenic release of CO_2 into the atmosphere. Sea surface temperatures have already increased by 1 °C since pre-industrial times (Hartmann et al. 2013) and rapid ocean warming poses a major threat to marine organisms (Hoegh-Guldberg and Bruno 2010; Pereira et al. 2010; Dawson et al. 2011; Pacifici et al. 2015; Urban 2015). In addition to mean global temperature increases, warming is resulting in more frequent and extreme summer heatwave events (Hughes et al., 2017). Tropical reef-building corals form an obligate endosymbiosis with dinoflagellate microalgae in the family Symbiodiniaceae (LaJeunesse et al. 2018). This symbiosis is sensitive to changes in temperature, where only small increases can trigger coral bleaching; the breakdown of the symbiosis between the coral host and its Symbiodiniaceae. The Symbiodiniaceae provide the coral host with majority of their energy through the translocation of photosynthetically-derived carbon (Muscatine and Porter 1977; Falkowski et al. 1984; Muscatine 1990), thus a prolonged disruption in the symbiosis can ultimately result in coral starvation and mortality. Since records began in 1980, three global coral bleaching events have occurred as a result of increasing sea surface temperatures, resulting in mass mortality and loss of coral cover (Hughes et al., 2017). The time between bleaching events is now half of what it was and thus corals have a shorter recovery window before the next bleaching event (Hughes et al., 2018). Furthermore, La Niña events that are associated with cooler periods, are now warmer than El Niño events of the past (Hughes et al., 2018). Thus, the intensity and frequency of extreme heating events are increasing.

Coral thermal tolerance limits depend not only on the thermal tolerance of the host animal, but also on that of their symbiotic algae belonging to the family Symbiodiniaceae. Physiological differences in thermal tolerances between Symbiodiniaceae populations have been attributed to variations in the stability of photosynthetic membranes and their fatty acid composition (Tchernov et al. 2004; Díaz-Almeyda et al. 2011), variations in reactive oxygen species release and antioxidant capacity (McGinty, Pieczonka, & Mydlarz, 2012; Suggett et al., 2008) and repair rates to photodamaged photosynthetic machinery (Takahashi et al. 2009). However, very little is known about the transcriptomic response that underlies thermal tolerance in the Symbiodiniaceae. All but one study to date have shown a minimal transcriptomic response of Symbiodiniaceae under elevated temperature conditions (Leggat and Medina 2011; Putnam et al. 2013; Barshis et al. 2014; Krueger

et al. 2015). The one study to show thousands of differentially expressed genes involved a heat stress experiment where *Cladocopium goreaui* was exposed to 32 °C for 13 days exhibiting upregulated genes by ≥ 4-fold that were involved in molecular chaperone and ROS scavenging activities (Levin et al. 2016).

Here, I had a unique opportunity to investigate the underlying genes and pathways that are differentially expressed between a thermo-tolerant and thermo-sensitive population of *C. goreaui* that were derived from the same monoclonal population. Specifically, in **Chapter 2** I carried out a laboratory evolutionary experiment exposing *C. goreaui* to 80 generations (~2.5 yr) of elevated temperature (31 °C), while the counterpart wild-type population was maintained at ambient temperature (27 °C). At the end of the experiment, the wild-type cells transplanted into elevated temperature were unable to grow and photosynthesise, while thermally selected cells showed significant positive growth and photochemical traits were unaffected by elevated temperature. Using a comparative transcriptomic approach, I characterise the differences in gene expression that distinguish the selected and wild-type population responses to elevated temperature (31 °) after 21, 28 and 35 days.

3.3 Materials and methods

Experimental design

Cladocopium goreaui (strain identification SCF055-01.10) was isolated from the coral species *Acropora tenuis,* Nelly Bay, Magnetic Island, Australia (19°1006”S, 146°50060”E) and the culture was started from a single cell. Note that SCF055-01.10 is the strain used in **Chapter 2** and originated from the heterogeneous culture(before single-cell isolation) investigated in two other previous studies (Howells et al. 2012; Levin et al. 2016). Cells were cultured in temperature-controlled environmental chambers (Steridium, er-rh-500, Australia) at 27 °C and 65 ± 10 µmol photons.m^{-2}.s^{-1} (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle in for ~6 months in 0.2 µm filtered sterile culture media, Daigo’s IMK for Marine Microalgae (Nihon Pharmaceutical Co., Ltd) before undergoing a thermal selection experiment as detailed in **Chapter 2** (**Figure 3.1a**). After approximately 80 generations (2.5 years) of thermal selection at 31 °C, the derived strain (selected Symbiodiniaceae*,* SS) and parent strain (wild-type Symbiodiniaceae, WT) were subjected to a reciprocal transplant experiment (**Figure 3.1b**). Triplicate populations of the SS and WT cells were pre-acclimated for two weeks (up to 6 generations) under their own and reciprocal temperature conditions (WT@27, WT@31, SS@27 and SS@31) before being transferred into culture vessels (25 cm^2, Corning®, Sigma-Aldrich) containing 10 mL of fresh culture media at a starting cell density of

200,000 cells mL^{-1}. Cells were pre-acclimated to allow assessment of any sustained changes in gene expression, rather than a short-term plastic response, for detailed temperature data see **Chapter 2**.

Seven, 14 and 21 days after transferring to fresh media (representing 21, 28 and 35 days in each temperature treatment, including the 2 weeks pre-acclimation), replicate cultures were gently agitated to re-suspend and homogenise the cells and 3 mL of culture from each replicate was subsampled (corresponding to up to one million cells), immediately snap frozen in liquid nitrogen and stored at -80°C for downstream RNA extraction. Sub-samples were taken at the same time of day across each of the time points. The sampling time course of 21 – 35 days of exposure to elevated temperature was chosen for a few reasons. Firstly, I wanted to avoid investigating any changes in gene expression that may have occurred as a result of transferring cells into fresh media. Secondly instead of capturing shorter-term plastic responses (i.e. over hours or days) to elevated temperature I wanted to investigate possible sustained changes in gene expression that for the SS at elevated temperature may have represented an adaptive response. Lastly, the growth and photophysiological traits that I measured in **Chapter 1** could be directly compared to changes in gene expression on the days of sampling.

Upon thawing, cells were pelleted (3000 g x 5 min), media removed and pellets lysed in buffer containing β-mercaptoethanol by bead beating with approximately 0.3 g of 710–1,180 µm acid-washed glass beads (Sigma) for 90 s. RNA extraction was carried out using GenElute Single Cell RNA Purification Kit (Sigma) following the manufacturer's protocol. Total RNA was sent to the Australian Genome Research Facility for library preparation and sequencing with an Illumina Hiseq2500 (100 bp paired-end). Resulting read depths for each replicate sample were an average of 15,768,808 reads (min. 8,363,733, max. 23,532,793).

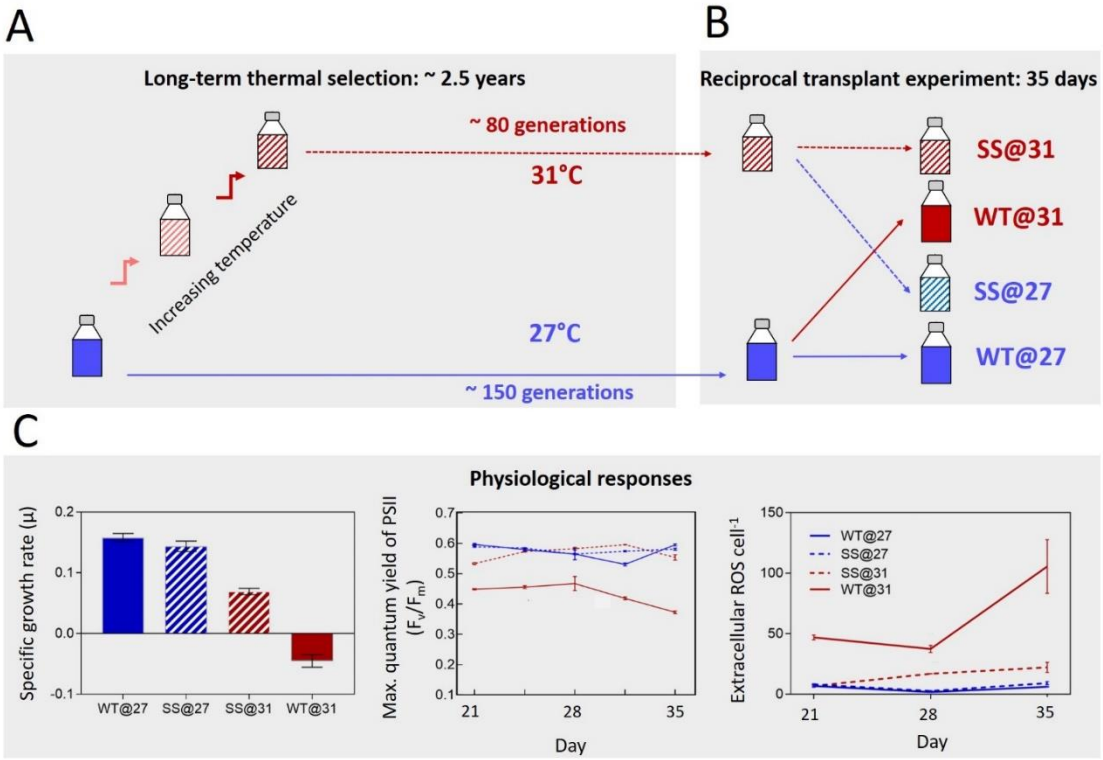

Figure 3.1. Experimental design detailing the long-term thermal selection experiment (A), reciprocal transplant experiment (B) and key physiological results (C), modified from **Chapter 2**). WT@27 represents the wild-type *Cladocopium goreaui* population that did not undergo any thermal selection, WT@31 represents the wild-type population transplanted to 31 °C for up to 35 days, SS@31 represents the long-term thermally selected population at elevated temperature and SS@27 represents the thermally selected population transplanted in the control temperature of 27 °C for up to 35 days. Specific growth and F_v/F_m values represent mean (±SE, n=6), Extracellular ROS $cell^{-1}$ values represent mean (±SE, n=3).

Transcriptome assembly

Raw, paired-end reads were trimmed with Illuminaclip (adapters Truseq3-PE.fa:2:30:10) using Trimmomatic (v. 0.36, Bolger, Lohse, & Usadel, 2014). Poly A/T tails were removed (min tail:6-A) and short (min length: 60 bp), low quality (min mean quality score: 20, base window: 1, base step: 1) and low complexity (dust method threshold: 7) sequences were removed using Prinseq-lite (v. 0.20.4, Schmieder & Edwards, 2011). Transcripts were assembled (minimum contig length 300 bp), using the standard Trinity (v.2.4.0) pipeline (Haas et al. 2013), into three transcriptomes; WT, SS and combined WT+SS. Redundant transcripts were collapsed into the longest representative transcript (99% sequence similarity over 99% of the shorter transcript) using cd-hit-est (v. 4.6.8, Y. Huang, Niu, Gao, Fu, & Li, 2010) for each assembly. The WT and SS transcriptomes were less than 1 % different in the number of total Trinity "genes" (**Appendix 3.1**) supporting the use of the one assembly transcriptome in further analyses. Furthermore, transcriptome completeness was assessed using BUSCO (v.3.0.2, Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) with Benchmarking universal single-copy orthologs from the Eukaryota dataset (odb9) with a default e-value cutoff for

BLAST searches of 0.01 and candidate regions to consider of 3. The combined transcriptome completeness was comparable to the individually assembled SS and WT transcriptomes (**Appendix 3.1**). To allow for direct comparisons between SS and WT gene expression, subsequent analyses utilized the combined transcriptome.

Differential gene expression, annotation and gene ontology analysis

In order to understand the cellular pathways that underlie differences in diverged physiologies, I investigated the comparative transcriptomic response of wild-type (WT, i.e., non-selected) *C. goreaui* under short-term elevated temperature (WT@31), thermally selected *C. goreaui* at elevated temperature (SS@31) and thermally selected *C. goreaui* transplanted back into the control temperature (SS@27) (as shown in **Chapter 2**, **Figure 3.1c**).

First, transcript abundance was estimated through the alignment-based method RSEM (v. 1.3.0, Li & Dewey, 2011). Using the gene-level abundance estimates for each replicate, a matrix of counts and normalised expression values was created. Normalisation takes into account transcript expression, transcript length and number of reads mapped to the transcript. Differentially expressed genes were identified between planned, pairwise comparisons, using edgeR, with a dispersion of 0.1, recommended for analyses if there are less than three replicates for a treatment (Robinson et al. 2010). Planned comparisons involved those genes differentially expressed between the WT@27 (control) with WT@31, SS@31 and SS@27 at each of the three time points. Differentially expressed genes (DEGs) were extracted that were at ≥ 8-fold differentially expressed with a false discovery rate (FDR) of ≤ 0.001 in any of the pairwise comparisons. A high fold minimum expression cut-off, based on log-ratio - mean-average (MA) and volcano plots (**Appendices 3.4-3.6**), combined with a conservative FDR reduced the number of marginally expressed transcripts.

Long open reading frames (ORFs) were identified that were at least 100 amino acids long and likely coding sequences were identified using TransDecoder (v.2.0.1). All ORFs were scanned for homology to known proteins using BlastP and BlastX searches (NCBI BLAST+, v. 2.7.1) and Pfam (v. 31.0) to search the peptides for protein domains. Search results were integrated to a SQLite (v. 3.23.1) database and an annotation report generated using Trinotate (v.3.1.1). A total of 33% of transcripts were annotated (**Table 3.1**), results that are in line with previous transcriptome annotations for *C. goreaui* (Levin et al., 2016, **Table 3.1**). Gene ontology enrichment was run on differentially expressed genes using GOseq (Young et al. 2010) with a fold change ≥8 between the pairwise comparisons. Only enriched GO categories that were supported by > 3 DEGs were retained.

3.4 Results and Discussion

The assembled transcriptome of *C. goreaui* comprised 124,187 genes, with average transcript length of 1093 bp (**Table 3.1**), results that are in line with previously published Symbiodiniaceae transcriptomes (Bayer et al. 2012; Parkinson et al. 2016; Levin et al. 2016). Furthermore, transcriptome completeness, assessed with benchmarking universal single-copy orthologs for eukaryotes (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015), was high at 95% (**Table 3.1**).

Table 3.1. Sequencing, assembly and annotation statistics for *Cladocopium goreaui* de novo transcriptome. Raw assembly refers to unfiltered Trinity assembly. Representative sequences refer to the processed Trinity assembly where redundant transcripts were collapsed into the longest representative transcript (99% sequence similarity over 99% of the shorter transcript) using cd-hit-est.

		Raw assembly	Representative sequences
Assembly	Total trinity "genes"	124,243	124,187
	Total Trinity "transcripts"	287,352	266,077
	Percent GC	52.57	52.24
All transcript contigs	N50	1,738	1,563
	Mean length	1,216	1,093
	Median length	858	744
	Assembled bases	349,437,378	247,050,504
Longest isoform per "gene"	N50	1,508	1,508
	Mean length	1,002	1,002
	Median length	615	615
	Assembled bases	124,468,611	124,429,598
BUSCO analysis	Complete BUSCOs	238 (78.5%)	287 (94.7%)
	Complete and single-copy BUSCOs	94 (31.0%)	139 (45.9%)
	Complete and duplicated BUSCOs	144 (47.5%)	148 (48.8%)
	Fragmented BUSCOs	31 (10.2%)	13 (4.3%)
	Missing BUSCOs	34 (11.3%)	3 (1.0%)
	Total BUSCO groups searched		303
Annotated transcripts	SwissProt (Blastx)		2,880 (1.1 %)
	SwissProt (Blastp)		67,918 (26.2 %)
	Pfam		77,638 (30.0 %)
	Eggnog		52,229 (20.1 %)
	KEGG		59,782 (23 %)
	TOTAL		85,747 (33.1%)
	GO terms (blast)		65,580 (25.3 %)
	GO terms (pfam)		47,023 (18.1 %)

Significant transcriptome-wide changes in gene expression were observed between the WT@27 (control) with the WT@31, SS@31 and SS@27 (**Figure 3.2, Appendices 3.2-3.5**) that included ≥ 8-

fold-changes in expression. Past studies have mostly observed only small changes in gene expression for members of the Symbiodiniaceae under different environmental conditions, including less than 2-fold different expression (Leggat et al. 2011; Gierz et al. 2017) or no changes in the gene expression response (Barshis et al. 2014). Other dinoflagellates have shown similarly low differential gene expression levels (Okamoto and Hastings 2003; Van Dolah et al. 2007). However, in line with my high levels of differential gene expression, Levin et al. (2016) identified genes that were ≥4-fold differentially expressed between two temperature treatments for two strains of *C. goreaui*, including the heterogeneous culture that the *C. goreaui* used in this study was isolated from.

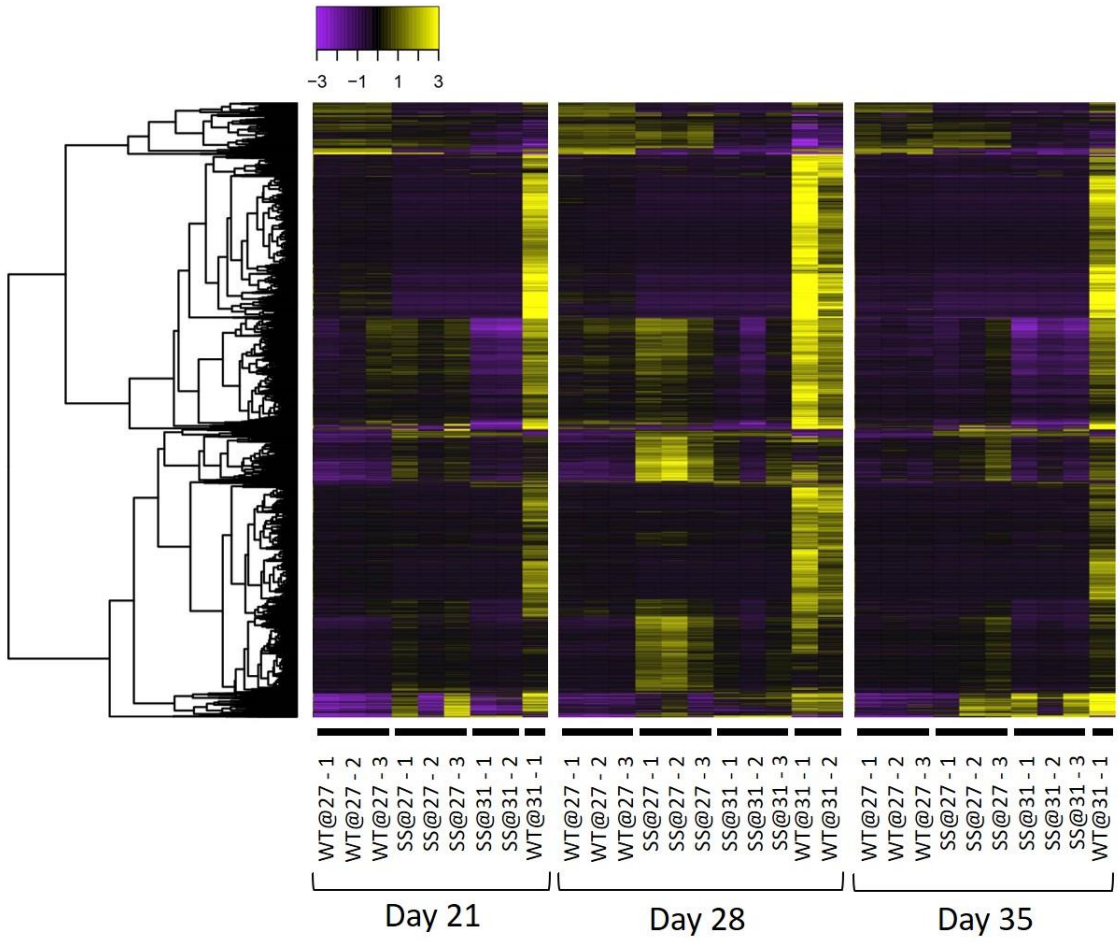

Figure 3.2. Hierarchical clustering of transcriptome-wide differentially expressed genes between samples and replicates in this experiment. Scale ranges from purple representing -3 log2 fold-change (-8 fold-change) expression of genes to yellow representing +3 log2 fold-change (+8 fold-change) in gene expression. Black represents no significant change in gene expression. Gene expression is represented by between one and three replicates for each population due to unsuccessful library preparation due to low RNA yields for some of the populations.

Response of short-term thermal stress on *C. goreaui* (WT@31)

Differential gene expression

Across the three sampling days, the WT@31 differentially expressed 631 (day 21), 2794 (day 28)and 204 (day 35) genes (≥ 8-fold, FDR ≤ 0.001) compared to the control (WT@27). At each time point the majority of WT@31 genes were upregulated rather than downregulated (**Figure 3**, **Appendix 3.2**), varying from 343 (54 % of DEGs) at day 21, to 2689 (96 %) at day 28 and 192 genes (94 %) at day 35 (**Appendix 3.2**). Similarly, the heterogeneous WT population of *C. goreaui,* from which the WT clonal strain was originally isolated , upregulated the majority (63%) of DEGs after 13 days of heat stress (Levin et al. 2016) but was accompanied by no apparent physiological stress. In contrast, after 21 days the WT@31 used here exhibited reduced growth, less efficient photosynthesis and showed high levels of the leakage of reactive oxygen species (i.e., extracellular ROS) as shown in **Chapter 2** (**Figure 3.1c**). These negative physiological responses to elevated temperature, along with an order of magnitude drop in DEGs from 2794 to 204 by the end of the experiment, suggest a failed acclimation response for this population to more than 21 days of exposure to elevated temperature. A potential lack of genetic variation as a result of the monoclonal nature of my population (although de novo mutations have likely accumulated over 2.5 yr of independent evolution) may have contributed to the lack of short-term resilience to temperature stress.

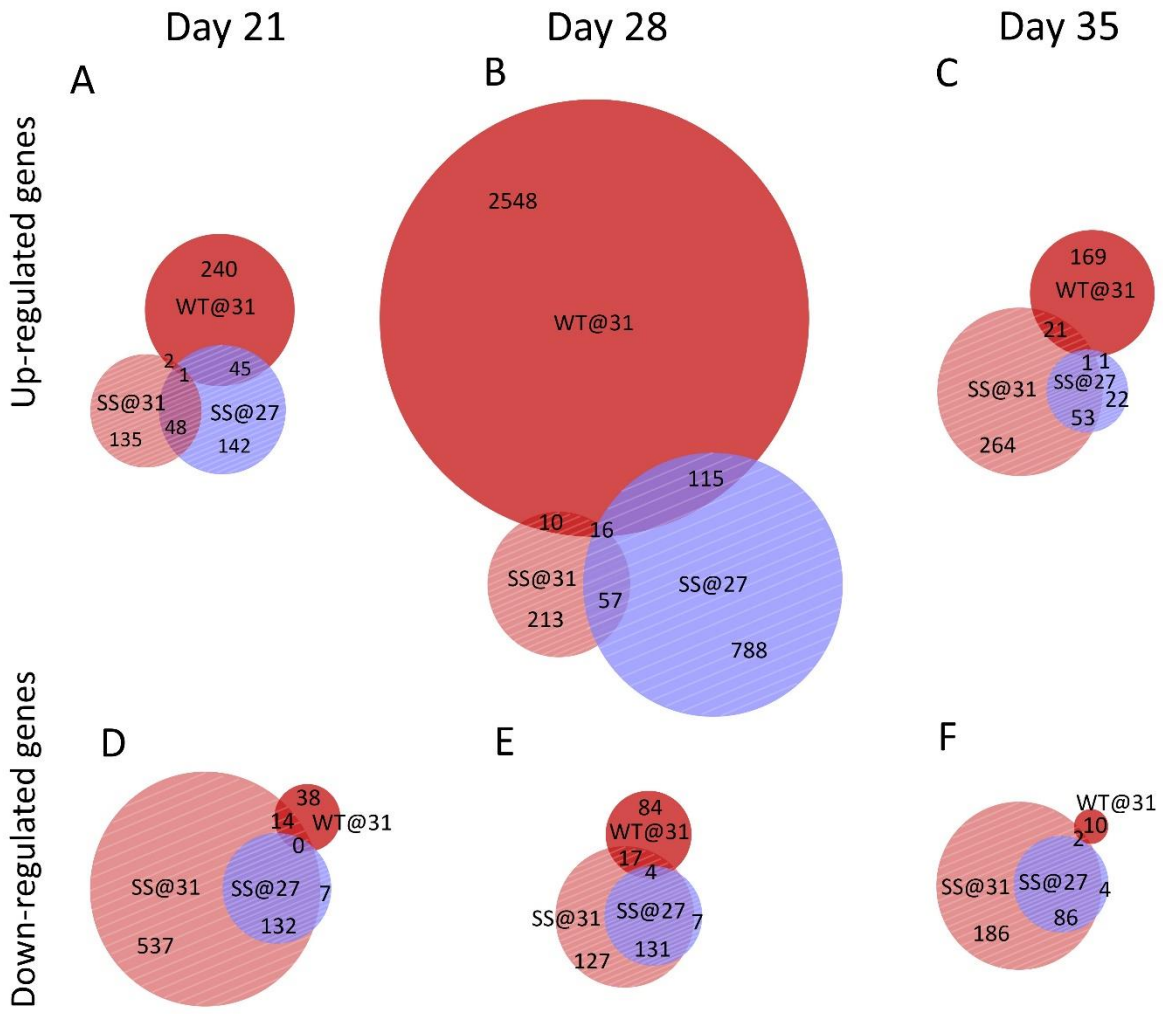

Figure 3.3. Venn diagram showing number of shared and non-shared upregulated (A-C) and down-regulated (D-E) genes (≥ 8-fold, FDR ≤ 0.001) between the short-term heat stressed *C. goreaui*, (WT@31), thermally-selected *C. goreaui* (SS@31) and the SS transplanted into the control temperature (SS@27), across three time points. Plots are made with BioVenn (Hulsen et al. 2008).

Upregulated genes and functional pathways

Gene ontology enrichment (GO) analysis revealed functional sets of genes that were involved in the stress response of the WT exposed to 31 °C. After 21 days, 123 gene ontology (GO) categories were enriched (FDR cut-off 0.01) for upregulated genes that fell mostly into the broader biological process categories of translational elongation and actin filament organisation (**Figure 3.4**). Actin (*ACT1*) and genes implicated in actin cytoskeleton organisation such as those encoding calmodulin (*CALM1*), and gelsolin-like proteins, were among the top five upregulated DEGs by the WT@31 (**Figure 3.7**). Actin is one of the main elements of the cytoskeleton and is a redox-sensitive system where reactive oxygen species (ROS) can cause actin cytoskeletal rearrangements, even playing a protective or regulatory role under high levels of oxidative stress (Gourlay and Ayscough 2005; Farah et al. 2011). The upregulation of actin and related genes under heat stress has been observed across taxa such as

mussels (Lockwood et al. 2010), fish (Buckley et al. 2006; Evans and Somero 2008), Arabidopsis (Fan et al. 2016) and coral (Desalvo et al. 2008). The WT produced significantly higher levels of extracellular ROS at 31 °C (**Chapter 2**, **Figure 3.1c**) and the upregulation of actin and related genes could represent an attempt at a protective mechanism.

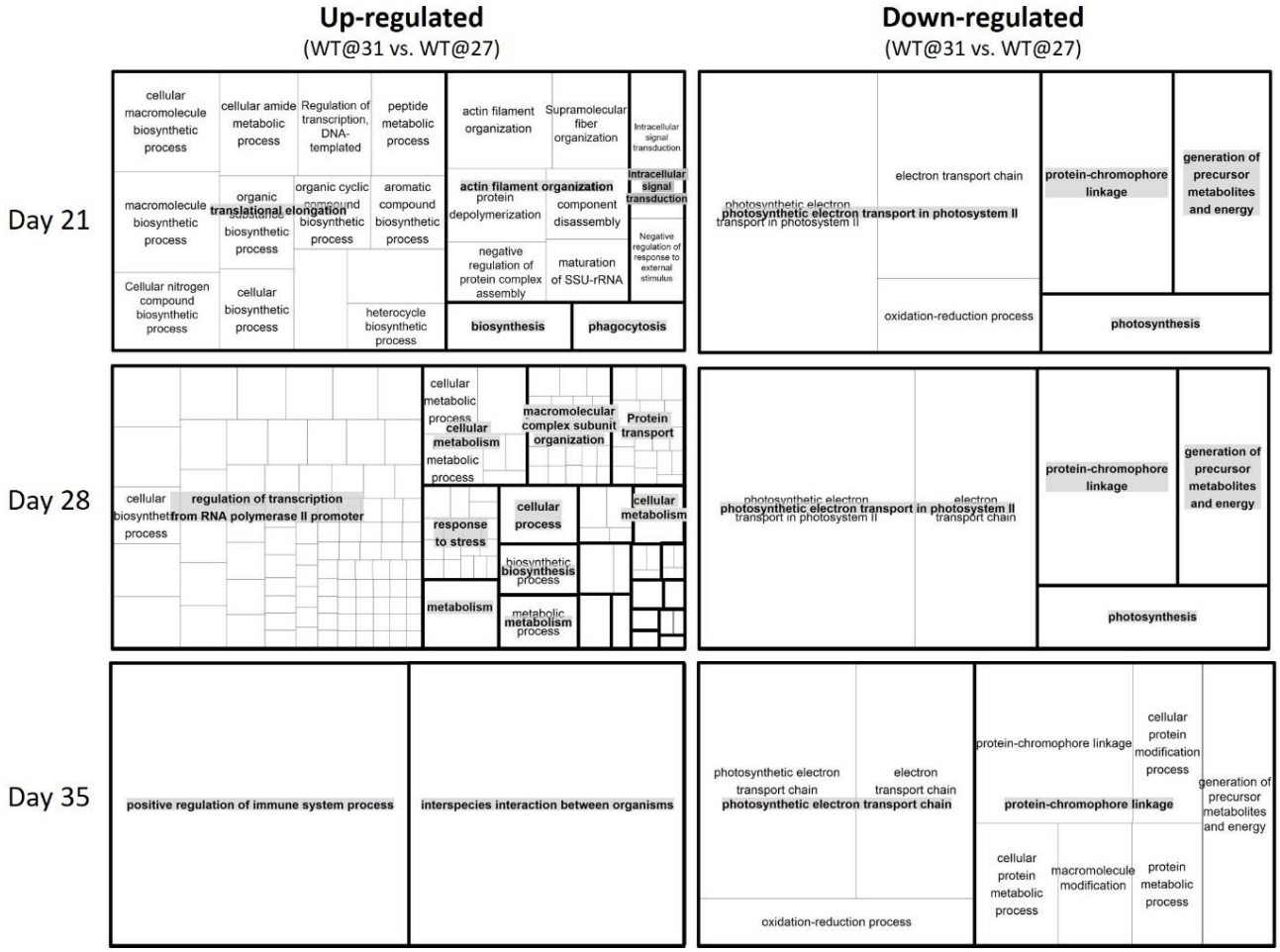

Figure 3.4. Treemap of summarised biological process Gene Ontology terms enriched for upregulated and downregulated biological processes for the WT@31 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Treemaps were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.

By day 28, 883 GO categories were enriched with most falling into the broader biological process categories of the regulation of transcription, metabolism, macromolecular complex subunit organization, protein transport as well as a response to stress (**Figure 3.4**). Actin filament-based processes remained among the most enriched GO categories for biological processes on day 28 and were supported by the enriched molecular function of “actin binding” (GO:0003779, **Appendix 3.6**).

In addition to its potential protective role against excess ROS, increased actin turnover and stabilisation has also been linked to the onset of apoptosis or programmed cell death in eukaryotic cells spanning many taxa (Posey and Bierer; Odaka et al. 2000; Gourlay and Ayscough 2005; Franklin-Tong and Gourlay 2008). Indeed, by day 28, three GO categories were enriched that contained 108 upregulated genes involved in programmed cell death/apoptosis. The programmed cell death pathway has been observed before in the Symbiodiniaceae and their hosts under heat stress (e.g. Desalvo et al., 2008; Mohamed et al., 2016; Zhou, Liu, Wang, Luo, & Li, 2019) and could be a pseudo-altruistic attempt at protecting host cells (in symbiosis) and the remaining algal population under stress (Arnoult et al. 2002; Huettenbrenner et al. 2003; Dunn et al. 2004). Cell death by the WT@31 was observed in **Chapter 2** through negative growth and deceasing cell abundances and from day 28, photosynthetic efficiencies also started to decline (**Figure 3.1c**), further supporting the upregulation of the programmed cell death pathway by the WT@31 and the decline in overall health of the cells.

Before initiating the programmed cell death pathway, a cellular response to stress is to recruit processes such as molecular chaperoning, protein repair, protein degradation and DNA repair (Martindale and Holbrook 2002; Kültz 2005). The WT@31 upregulated all of these processes on day 28, but not days 21, or 35. Firstly, chaperonin activity acts to assist with protein folding and has been associated with thermal stress that can result in misfolded, slow rates of assembly or biosynthetic errors of proteins (Wang et al. 2004). I observed 57 DEGS supporting the enriched GO category of protein folding on day 28 (GO:0006457). Chaperone-like activity has been observed in Symbiodiniaceae under heat stress in some studies (Rosic et al. 2011a, b; Ladner et al. 2012; Levin et al. 2016) while others report no notable differences (Leggat et al. 2011; Putnam et al. 2013; Barshis et al. 2014; Krueger et al. 2015). Secondly, protein degradation may be necessary to prevent the toxic build-up of damaged proteins in a cell. The gene *MUG145* was among the top upregulated genes by the WT@31 on day 28 (**Figure 3.7**). *MUG145* encodes a RING finger protein that is a mediator of ubiquitin ligase activity (Joazeiro and Weissman 2000), part of a pathway that acts to degrade and clear damaged protein from subcellular compartments (Parsell and Lindquist 1993; Gottesman et al. 1997; Pickart 1999; Wilkinson 1999). Indeed 14 ubiquitin-associated enriched GO categories, supported this activity on day 28. Finally, histone H4 (*HIST1H4A*) was among upregulated genes by the WT@31 and was among the top five on day 21 (**Figure 3.7**). Histone proteins can play a protective role as part of the defence against DNA damage by oxidation in the nucleosome (Ljungman and Hanawalt 1992; Ramirez-Parra and Gutierrez 2007). Supporting this process, 11 GO categories were enriched for processes involving histones on day 28 by the WT@31 and seven GO

categories were enriched that directly involved a response to DNA damage, supported by 101 upregulated DEGs.

Notably, 25 GO categories were enriched for genes involved in fatty acid/lipid metabolism, catabolism and transport on day 28 and these were supported by 521 upregulated DEGs. Notably none of these categories were enriched for upregulated genes by the SS@31. Indeed, amongst the top upregulated genes for the WT@31 were those that have functions in lipid/fatty acid metabolism and transport, i.e. genes encoding the electron transfer flavoprotein subunit alpha 2 (*ETFA*) and polyketide biosynthesis protein (*pKsE*), plasma membrane choline transporter (*PNS-1*) and long-chain fatty acid CoA ligase (*ACSBG2*) genes; Changes in lipid metabolism have been observed across many taxa exposed to elevated temperature (Imbs and Yakovleva 2012; Yampolsky et al. 2014) and indicates a change in membrane and lipid storage composition. The energetic costs of living at elevated temperature are comparatively greater and thus the metabolism of lipid reserves is not unexpected. Additionally, lipid membrane damage occurs across all major taxa in response to stress (Parasassi et al. 1991; Steels et al. 1994; Zeng et al. 1999). For the Symbiodiniaceae the degree of lipid saturation in the chloroplast thylakoid membranes can be a key determinate of the sensitivity to heat (Hillyer, Tumanov, Villas-Bôas, & Davy, 2016; Tchernov et al., 2004; Warner et al., 1999). The upregulation of long chain fatty acid synthesis and transport may be an attempt to alter membrane lipid composition in response to stress, by the WT@31.

By day 35 only five GO categories were enriched for upregulated genes that fell into the broader biological processes of positive regulation of immune system processes and interspecies interaction between organisms (**Figure 3.4**). The lack of sustained gene expression and reduction in enriched GO processes suggests an inability for the WT to acclimate to elevated temperature perhaps due to an inability to repair and/or remove damaged cellular molecules as a result of increasing ROS levels. Indeed, increasing levels of extracellular ROS in the WT@31 was clear from the in **Chapter 2** (**Figure 3.1c**).

Downregulated genes and functional pathways

On days 21 and 28, there were far fewer GO categories that were enriched for downregulated genes (22 and 21 categories) than for upregulated genes (123 and 883 categories). Most enriched categories were involved in core photosynthetic machinery that were shared across all three time points. The most downregulated genes included those coding for the cytochrome b6-f complex (*petD*), Photosystem II CP43 (*psbC*), CP47 (*psbB*), photosystem I chlorophyll apoprotein (*psaA*) and D2 (*psbD*) protein. Photosynthetic ability is often used as an indicator of Symbiodiniaceae health

(e.g. Buxton, Takahashi, Hill, & Ralph, 2012) and the downregulation of photosynthesis in response to heat stress is a common phenomenon in thermo-sensitive cells (e.g. Baumgarten et al., 2013; Gierz et al., 2017; McGinley et al., 2012; Ragni et al., 2010; Robison & Warner, 2006). The photosynthetic machinery is sensitive to heat and the downregulation of such genes is likely a mechanism to reduce the production of ROS in the chloroplast to prevent further damage to photosynthetic apparatus and other cellular components (Lesser, 2006; Murata et al., 2007). Alternatively, their downregulation may be an attempt to conserve remaining energy for other vital cell processes (McGinley et al. 2012).

Response of *C. goreaui* (SS@31) to long-term thermal selection

Differential gene expression

The thermally selected *C. goreaui* (SS@31) exhibited a more stable transcriptomic response than the WT@31. The SS@31 differentially expressed 872, 575 and 613 genes across the three sampling days (**Figure 3.3**, **Appendix 3.2**). Unlike the WT@31, the majority of these genes were downregulated at the first sampling point of day 21 (686 genes, 79% of DEGs). The remaining two sampling points were comparable, with 279 (49%) and 274 (45%) downregulated DEGs, at days 28 and 35 respectively (**Figure 3.3**, **Appendix 3.2**). An increased proportion of downregulated DEGs in response to elevated temperature has previously been observed in marine organisms including the Symbiodiniaceae and corals (Baumgarten et al., 2013; Bay & Palumbi, 2015, Levin et al., 2016). This pattern of downregulation was also described in two heat tolerant *Daphnia* (Yampolsky et al. 2014), yeast (Causton et al. 2001) and *Drosophila* (Levine et al. 2011) at elevated temperature. The SS performed much better physiologically than the WT at 31 °C (**Chapter 2**, **Figure 3.1c**), exhibiting positive growth rate, and similar photophysiology and extracellular reactive oxygen species levels to the control.

Upregulated genes and functional pathways

Gene ontology enrichment (GO) analysis for the SS@31 revealed 61, 80 and 106 GO categories enriched for upregulated genes on days 21, 28 and 35, respectively. Across each time point enriched biological processes fell into the broader category of DNA-templated transcription, initiation (**Figure 3.5**), while for enriched molecular functions, transcription factor activity involved in RNA, DNA and protein binding, as well as sigma factor activity were upregulated at each time point (**Appendix 3.7**).

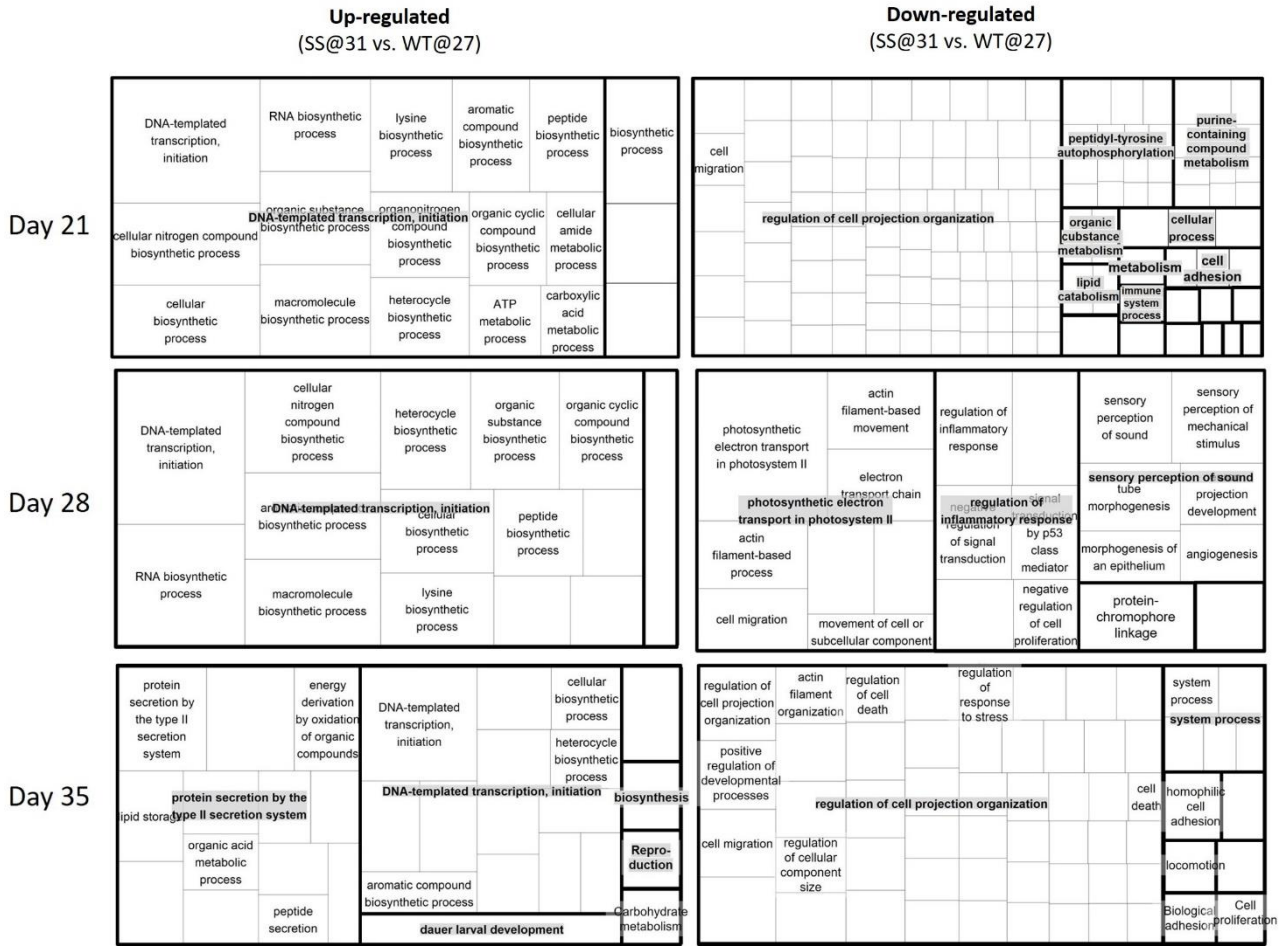

Figure 3.5. Treemap of summarised biological process Gene Ontology terms enriched for upregulated and downregulated biological processes for the SS@31 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Summary and treeplots were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.

Among the top five most expressed genes were those supporting enriched transcription factor activities including those encoding the cellular nucleic acid-binding protein (*CNBP*) and UDP-N-acetylglucosamine (*OGT*) (**Figure 3.7**). Transcription factors are proteins that can activate or deactivate specific genes and their induction or repression can play a crucial role in converting the perception of abiotic stress into stress-responsive gene expression. They can result in the activation of whole networks of genes that act together to enhance stress tolerance as has been observed in plants (Chen and Zhu 2004; Xu et al. 2008; Akhtar et al. 2012; Guo et al. 2016). While around 7% of plant genomes are assigned to transcription factors (Baniwal et al. 2004; Udvardi et al. 2007) and up to 9% of transcription factor genes make up higher eukaryotic transcriptomes (Bayer et al. 2012), only up to 0.27% of the Symbiodiniaceae genome consists of transcription factors (Bayer et al. 2012; Shoguchi et al. 2013). Similarly, only a small proportion of the transcriptome, at 0.71%, contains transcripts annotated with GO blast matches to “transcription factor”. Despite this, ‘DNA-templated transcription, initiation’ is the mostly significantly enriched GO category for the SS@31 across all

three time points. While transcription factor activity was also enriched in the WT@31, this was not sustained and enriched only at the middle time point. Thus, the sustained regulation of such genes may be vital to allowing the SS@31 to cope with elevated temperatures. Indeed epigenetic effects have been found in other organisms (Artemov et al. 2017; Metzger and Schulte 2017) and the targeting of genes encoding transcription factors has been discussed as genetic manipulation tool in crops to enhance stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2006; Agarwal et al. 2006; Lata and Prasad 2011). For the Symbiodiniaceae, such genes could be important targets for enhancing thermal tolerance.

For the SS@31 population, there was no consistent upregulation of genes encoding chaperone, antioxidant or heat shock proteins over time that may be expected of sustaining thermal tolerance. Indeed, 16 genes involved in the response to oxidative stress were downregulated on day 28 while only three genes were upregulated on day 28 that functioned in the chaperone complex. Surprisingly, a gene encoding the type II secretion system protein was among of the five most upregulated genes by the SS@31 at each time point. The T2SS pathway was first discovered in the bacterium, *Klebsiella oxytoca* (d'Enfert et al. 1987) and to date, genome studies indicate that this system is present in hundreds of bacterial species belonging to the Proteobacteria as well as other major bacterial groups (Nivaskumar and Francetic 2014). Type II protein secretion is a process used to secrete proteins such as toxins, proteases and lipases into the surrounding environment or into target cells and is generally considered pathogenic (Sandkvist 2001). Sequencing methods in this experiment targeted polyA-tailed mRNA specific to eukaryotes, although the polyadenylation of mRNA in prokaryotes has been reported (Sarkar 1997) and thus the occasional occurrence of bacterial transcripts with this method is possible. However, T2SSs are also associated with non-pathogenic bacterial symbionts of plants and animals, including the leech symbiont *Aeromonas veronii* (Maltz and Graf 2011), plant symbionts, *Gluconacetobacter diazotrophicus* (Arrieta et al. 2004) and squid symbiont *Allivibrio fischeri* (Evans et al. 2007, 2008).

Upon aligning the T2SS transcript with a published *Cladocopium* genome (Liu et al. 2018), SymbC1.scaffold21.313 was the closest match at 40 % similarity. d an evalue of 6e-73 and the scaffold contained a gene with a similar annotation to ours; e.g. type II secretion system protein GspE . The T2SS transcript when BLASTx searched against NCBI (used to search for a nucleotide sequence in an amino acid database), matched most closely to bacterial genomes with a similarity of up to 50 % and an e-value of 4e-124, values that are slightly higher than those for the *Cladocopium* genome.

The cultures used in **chapter 2** were not axenic and cultured strains of the Symbiodiniaceae have been found to typically harbour abundant bacterial communities (Lawson et al. 2018). It is possible that thermal selection acted upon both the Symbiodiniaceae and a potentially mutualistic bacteria, where intercellular interactions, perhaps through secretion, aids the growth and survival of selected cells. Notably the type II secretion protein was not up- or down-regulated by the heat-sensitive WT@31, perhaps supporting the exclusivity of this function to the SS population to cope with heat stress. Due to their high stability, T2SS are found among bacteria that are adapted to extreme conditions (Worden et al. 2006; Evans et al. 2007; Parrilli et al. 2008), supporting their upregulation at elevated temperature in this study. If the transcript in question is of bacterial origin being closely associated with the SS@31 population, the presence of such transcripts raises questions about the interactions of co-existing microbe interactions and their potential role in thermal tolerance (Lawson et al. 2018). If this transcript is a homologous gene upregulated by the SS@31 Symbiodiniaceae, it could be a gene key to the thermal tolerance of the selected Symbiodiniaceae cells under heat stress and should be studied further.

Downregulated genes and functional pathways

The enriched GO categories for genes downregulated by 8-fold or more by the SS@31 were greater than those GO categories enriched for upregulated genes at 1056, 93 and 247 on days 21, 28 and 35, respectively. The first and last time points shared many common enriched biological processes that included the broader categories of the regulation of cell projection organization (**Figure 3.5**) that encompassed the regulation of lamellipodium and filopodium assembly and organisation (cytoplasmic projections extending from the periphery of a migrating or moving cell), cell migration and motility as well as cell proliferation and cell differentiation. A recent study showed a 50% reduction in cell motility in another species of the Symbiodiniaceae (formerly ITS2 type clade A1) at 31 °C (Nitschke et al. 2015), and for other free-living dinoflagellates drastic declines in swimming speeds have also been observed at elevated temperature (Kamykowski and McCollum 1986). The downregulation of such processes is likely an attempt to reduce energy expenditure in an energetically costly environment, however decreased motility may have wider-reaching implications for cell dispersal, migration in the water column or the onset of symbiosis with a host and could be a cost of living under elevated temperature.

On day 28, the SS@31 shared most of the enriched biological processes with days 21 and 35 but in addition photosynthetic electron transport processes were also enriched for downregulated genes (**Figure 3.5**) that included five enriched GO categories and 24 DEGS. Despite this, there were no

photosynthetic-related genes among the top 5 most downregulated genes on day 28 and by day 35 there were no longer any enriched GO categories for downregulated photosynthetic genes. Indeed, physiological data presented in **Chapter 2** revealed no apparent cost of elevated temperature to measured photophysiological traits (**Figure 3.1c).** Additionally, the regulation of inflammatory response was enriched for downregulated genes on day 28 (**Figure 3.5**). A gene encoding peptidase_C14 that represents a subunit found in caspases, was one of the top five most downregulated genes on day 28, as well as day 35 (**Figure 3.7**). Caspases are proteases that play a role in inflammation and apoptosis (Thornberry and Lazebnik 1998). The downregulation of programmed cell death/apoptosis occurred at days 21 and 35 had 25 and six enriched GO categories supported by 310 and 77 DEGs, respectively. The downregulation of the programmed cell death pathway was in direct contrast to the upregulation of such processes by the WT@31.The downregulation of other processes and genes by the SS@31 also contrasted with the WT@31. This included chaperonin activity where one of the top most downregulated genes by the SS@31 was peptidyl-prolyl cis-trans isomerase (Fkbp2) on day 35, which was one of the most upregulated genes by the WT@31 on day 35. In addition, Long-chain-fatty-acid--CoA ligase and GTP cyclohydrolase 1 were amongst those genes highly upregulated by the WT@31 and downregulated by the SS@31 (**Figure 3.7**).

Summary of up and down-regulated genes and functional pathways (SS@31 vs WT@27)

The stable upregulation of transcription factors over time by the SS@31, the lack of upregulated genes associated with a physiological stress response and the consistent downregulation of many genes represent marked differences with the WT@31, suggesting that these factors may be the causative mechanism of thermal tolerance in the SS. Furthermore, the upregulation of a gene homologous to a bacterial T2SS by the SS@31 could be important for its thermal tolerance, especially when these genes were not found significantly up or downregulated by the WT. The origin of this gene (bacterial or Symbiodiniaceae) should be further investigated.

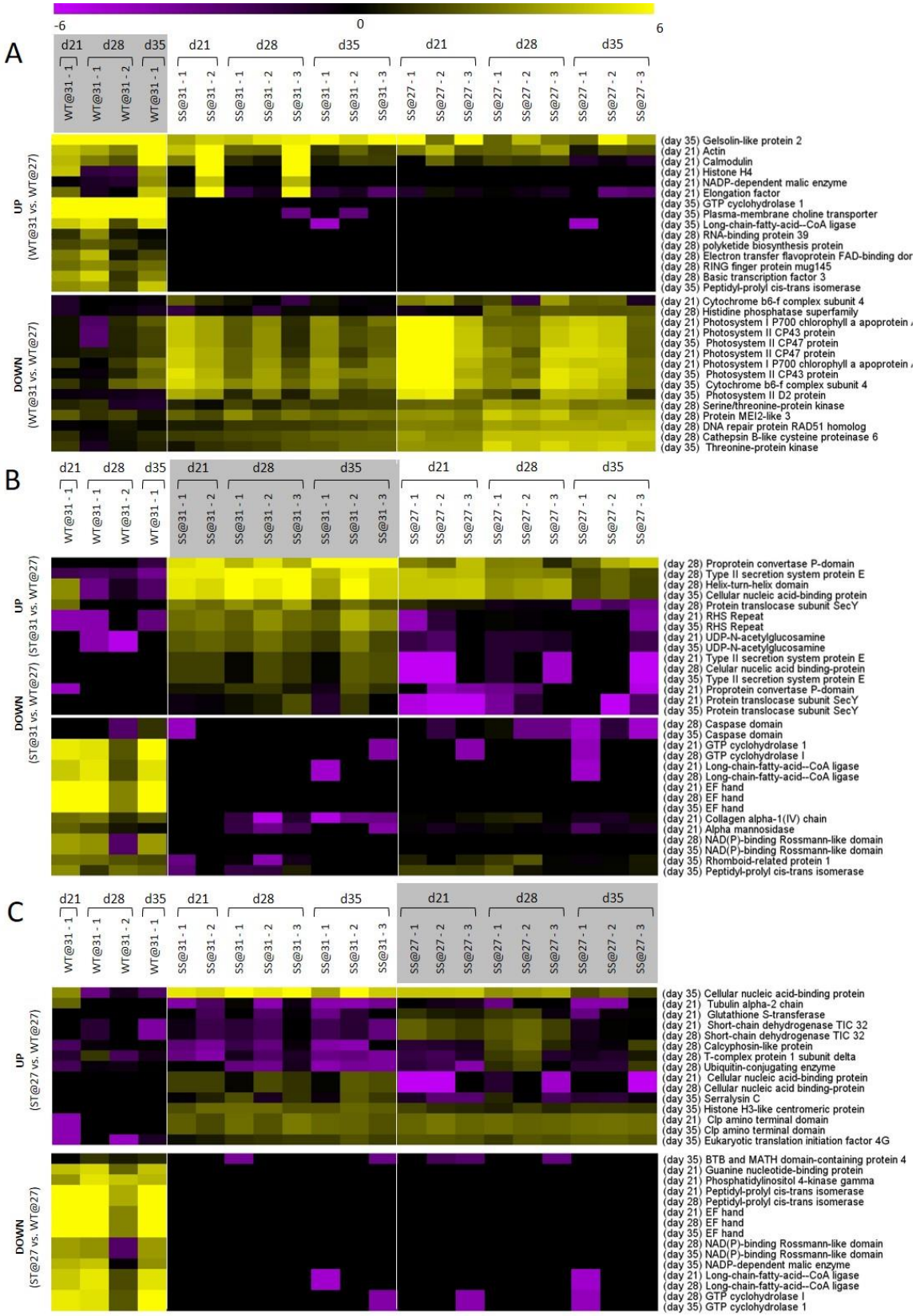

Figure 3.7 Top five differentially expressed upregulated and downregulated, annotated genes (DEGs) by (a) the WT@31 (b) SS@31 and (c) SS@27, at each time point (days 21, 28 and 35, listed to the right of each heatmap). Top DEGs were chosen based on the highest fold-change of each sample in question compared to the control, WT@27. Data are log2 transformed. Genes are hierarchically clustered with an Elucidean distance metric. Heatmaps were created with TM4 MeV (v. 4.9.0).

Response of the thermally selected *C. goreaui* transplanted to the control temperature (SS@27)

Differential gene expression

The thermally selected strain of *C. goreaui* transplanted to the control temperature (SS@27) differentially expressed 378, 1118 and 167 genes after 21, 28 and 35 days, respectively (**Figure 3**, **Appendix 3.2**). At the first two time points the majority of genes were upregulated rather than downregulated. The number of genes that were upregulated increased from 326 (62 % of DEGs) at day 21, to 976 (87 %) at day 28. Finally, by day 35 the numbers of up and downregulated genes were far fewer and more balanced, with 77 DEGs upregulated (46% of DEGs) (Figure 3, **Appendix 3.2**). Physiological data indicated the ability of the SS@27 to maintain growth, photosynthesis and extracellular ROS levels that were similar to the WT@27 control (**Chapter 2**, **FIgure 3.1c**). Thus, the high number and proportion of DEGs upregulated by the SS@27 on day 28, followed by an order of magnitude drop in DEGs by day 35 does not reflect a failed acclimation response, as observed for the WT@31. Instead it suggests that by the end of the experiment, a minimal transcriptomic response was required for the SS to function similarly to the WT at the control temperature, despite ~80 generations of thermal selection.

Upregulated genes and functional pathways

Differentially expressed genes fell into 52, 469, and 0 enriched GO categories for upregulated genes after 21, 28 and 35 days of the experiment, respectively. Most GO categories for enriched biological processes of upregulated genes fell into the broader category of translation on both days 21 and 28, with additional enriched categories that included cellular response to retinoic acid, cellular response to vitamin D and metabolic processes. Retinoic acid is formed through the oxidation of retinol (vitamin A) and both vitamin A and D can act as non-enzymatic quenchers of ROS (Montserrat et al. 2011). In line with this, enriched GO categories for upregulated molecular functions at both time points included glutathione transferase activity, oxidoreductase activity, and peroxidase activity (**Appendix 3.8**). Glutathione S-transferase (yghU) and Short-chain dehydrogenase (TIC32) are involved in antioxidant processes and were coded by genes that were among the top five most upregulated by the SS@27 on day 21 and the latter (TIC32) also on day 28 (**Figure 3.7c**). Neither genes were significantly upregulated by either the WT@31 or SS@31. Antioxidants such as these are common biomarkers of oxidative stress (Montserrat et al. 2011), and thus I did not expect their increased expression when the SS were placed into the control temperature, a presumably non-stressful condition. Indeed, extracellular ROS levels were not elevated by the SS@27 in **Chapter 2** (**Figure 3.1c**). It is possible that the transplantation of the SS into a relatively “new” environment, not

experienced for 80 generations, may have elicited a short-term stress response and a subsequent higher ROS production could have been masked by the successful upregulation of antioxidant genes on days 21 and 28.

Vitamins A and D are also regulators of gene expression by binding to specific receptors (Ross et al. 1993;.), acting as transcription factors. For example, retinoic acid, the metabolite of vitamin A (retinol), functions as a ligand for a family of nuclear retinoic acid receptors that regulate transcription of target genes (Rastinejad et al. 2013; Huang et al. 2014). Furthermore, among the top upregulated genes by the SS@27 (**Figure 3.7**) were others coding for transcription factor activities such as cellular nucleic acid-binding protein (CNBP), a transcription regulator, as well as the eukaryotic translation factor 4G (EIF4G) that aids the recruitment of ribosomes to mRNA (Gingras et al. 1999). Also likely involved in playing a regulatory role in gene expression was the gene encoding a histone H3-like centromeric protein (cpar-1) which was one of the most upregulated genes by the SS@27, exhibiting a 156-fold change compared to WT@27. Furthermore, among the top upregulated genes by the SS@27 were those involved in chaperonin-like activities such as the chaperonine T-complex protein 1 subunit delta (*CCT4*). Additionally, the Clp amino terminal domain was among the top upregulated genes and is found in the ClpA and ClpB proteases, thought to be involved in eliminating damaged proteins that cannot be rescued by chaperones, by unfolding them and delivering them to peptidases (Weber-Ban et al. 1999) or to reverse harmful protein aggregations (Schirmer et al. 1996; Zolkiewski et al. 1999; Glover and Lindquist 1998). The gene encoding a Ubiquitin-conjugating enzyme E2 8 (*UBC8*) was one of the most upregulated genes by the SS@27 exhibiting a 224 fold-change on day 28, suggesting a need to clear damaged proteins from subcellular compartments (Parsell and Lindquist 1993; Gottesman et al. 1997; Pickart 1999; Wilkinson 1999). However, by day 35 a BTB and MATH domain-containing protein 42 (*bath-42*) was one of the most downregulated genes likely involved in the ubiquitin pathway and protein degradation, suggesting a reduced need to remove damaged proteins. Furthermore, the lack of enriched GO processes by the end of the experiment also suggests that the SS was no longer under stress, responding similarly to the control; this is further supported by physiological data for the SS@27 where growth and photophysiology were not significantly different to the control WT@27 (**Chapter 2**) (**Figure 3.1c**).

Downregulated genes and functional pathways

Only 10 and four GO categories were enriched for downregulated genes by the SS@27 on days 21 and 28 and none by day 35. These collapsed into the broad biological function categories of actin

filament depolymerisation on day 21 and unconventional myosin complex on day 28 (**Figure 3.6**). Myosins are proteins that convert adenosine triphosphate (ATP) into mechanical energy, and have been associated with the regulation and organisation of the actin skeleton ((Kalhammer and Bähler 2000; Hartman et al. 2011). Both the SS@31 and SS@27 downregulated actin-related processes, while the WT@31 upregulated actin related processes. Indeed, the SS@31 and SS@27 showed similarities in the most downregulated genes including those encoding GTP cyclohydrase (*SPAC17A5.13*), that is involved in folate and biopterin pathways, Long-chain fatty acid CoA ligase (*ACSBG2*), an NAD(P)-binding Rossmann-like domain, Peptidyl-prolyl cis-trans isomerase (*PCKR1*) and an NADP-dependent malic enzyme (*ME1*) (**Figure 3.7**).

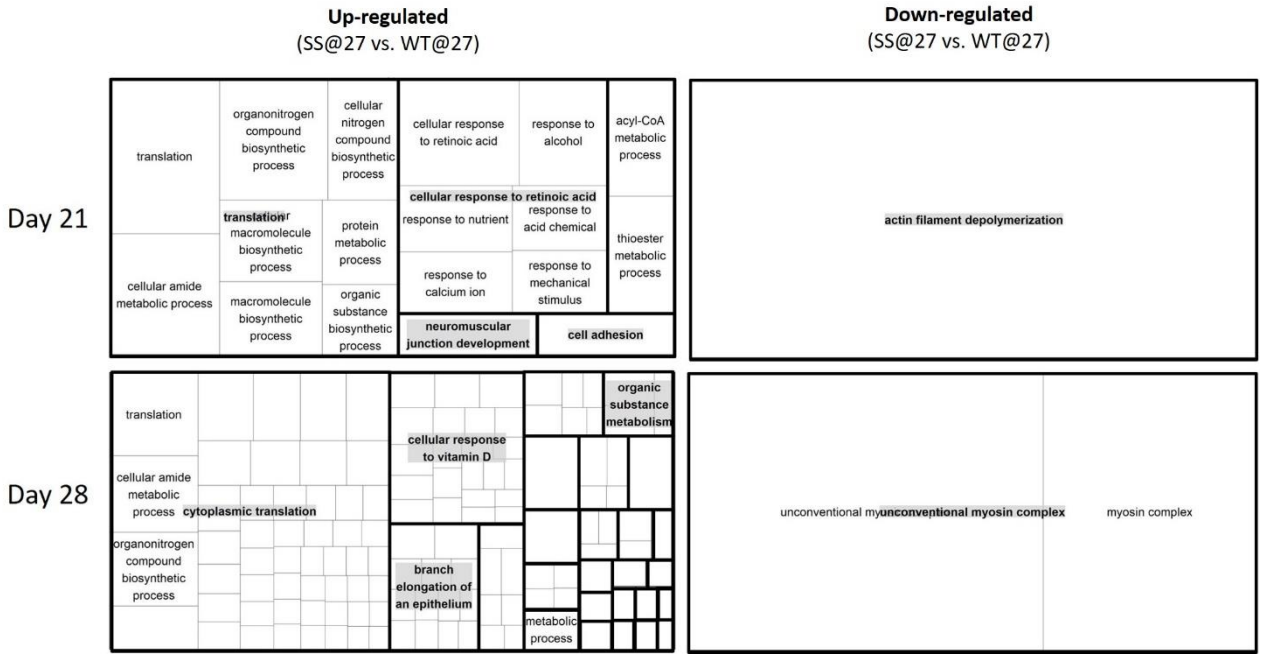

Figure 3.6. Treemap of summarised biological process Gene Ontology terms enriched for upregulated and downregulated biological processes for the SS@27 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Summary and treeplots were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.

The upregulation of genes involving the regulation of translation and post-translational modification including chaperone-like proteins as well as antioxidant genes is indicative of a stress response. However, the lack of significantly enriched GO categories by the end of the experiment, along with the ability of the SS@27 to grow, photosynthesis and maintain low levels of extracellular ROS that were not significantly different to the control (**Chapter 2**) (**Figure 3.1c**), suggests a positive acclimatory response of the SS@27. Alternatively, the few genes that were differentially expressed at the end of the experiment may have been enough to sufficiently protect the SS@27 in a "new" temperature condition. Nonetheless, my results do show that after 80 generations/2.5 years at

elevated temperature, the SS strain still mounted a considerable transcriptome response when placed back into the original control temperature; even after 35 days of residing in the control temperature, where 187 genes were differentially expressed at a log fold-change of ≥ 8 compared to the WT at 27°C control.

Summary and Conclusions

In this study I have characterised the transcriptomic response of (1) short-term heat stress on wild type *C. goreaui* (WT@31), (2) long-term thermal selection on *C. goreaui* (SS@31), and (3) the transplant of thermally selected *C. goreaui* (SS@31) back into the control temperature (SS@27). The WT@31 exhibited a classic stress response that included the upregulation of genes involved in the regulation of transcription, the actin cytoskeleton, chaperone activity, protein degradation pathways and programmed cell death. Only genes involved in photophysiology were consistently downregulated in WT@31 over the three sampling time points, while upregulated functional gene pathways were inconsistent over time. After 35 days of exposure to elevated temperature the transcriptomic response of the WT@31 was minimal. This, combined with a physiological decline (**Figure 3.1c**) that included cell death (negative growth) indicates that what I observe here is a failed, population acclimation response and transcriptome-wide shut down toward the end of the experiment. The long-term adapted SS@31 showed a very different and more stable transcriptomic response, exhibiting many of the same DEGs and enriched GO categories over time. The majority of enriched upregulated processes over time included transcription factor activity and the type II secretion pathway. Many genes were consistently downregulated by the SS@31 over time, including those involved in actin cytoskeleton, antioxidant activity, programmed cell death, motility, chaperonin activity and metabolism; responses associated with a stress response.

The consistent up- and especially downregulation of genes by the SS@31 but not by the WT@31 points to the causative mechanisms behind the comparatively high thermal tolerance of the SS cells. However, the downregulation of some processes, while perhaps aiding in thermal tolerance, may come at an ecological cost and should be investigated further. For example, reduced metabolism may result in less energy translocated to the host when in symbiosis, while reduced motility may affect the chance of interacting with a potential host and thus the onset of symbiosis. The bleaching response of coral recruits hosting either the SS or WT at elevated temperature was investigated in **Chapter 2** and despite the comparatively high thermal tolerance of the SS *in vitro*, coral recruits bleached to the same extent when hosting WT or SS, although the bleaching response was somewhat delayed in two of three coral species hosting the SS. Free-living populations of the

Symbiodiniaceae make up the crucial environmental pool of symbionts available for corals with horizontal uptake and thus identifying the thermal response of such populations is important. However, it is also important to study the response of the Symbiodiniaceae to heat in symbiosis where the coral host can have a large effect on their symbionts' physiology, and I suggest this as an important future research direction. Identifying genes and pathways that underly thermal tolerance could be targets for genetic manipulation experiments and used to develop thermally tolerant strains of the Symbiodiniaceae for use in coral restoration and conservation initiatives.

Chapter 4: Experimental evolution in coral photosymbionts as a tool to increase thermal tolerance

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4.1 Summary

Coral reefs are under major threat from ocean warming. When temperatures become too high corals bleach, expelling their symbiotic, photosynthetic microalgae (Symbiodiniaceae), which they depend on for much of their nutritional requirements. Prolonged bleaching has led to widespread coral mortality and the severity and frequency of bleaching events are predicted to increase in the future. Coral bleaching tolerance is influenced by the thermal tolerance of the Symbiodiniaceae harboured, and these microbial members of the coral holobiont may be able to evolve more rapidly than the coral host itself. Here, I examined the response of replicate cultures of five genetically distinct Symbiodiniaceae strains, belonging to four genera; *Symbiodinium*, *Durusdinium*, *Gerakladium* and *Fugacium* (formerly known as ITS2 types A3c, D1, G3 and F1) to increasing temperatures over the course of approximately one year. For three strains (belonging to *Symbiodinium*, *Gerakladium* and *Fucacium*), I observed a stable adaptive change at the end of this exposure period, which equated to only 41-69 asexual generations. The long-term selected Symbiodiniaceae culture replicates (SS) showed faster growth rates under short-term, acute heat stress, and in some cases higher photosynthetic efficiencies, compared to wild-type populations (WT). My results considerably extend the field of experimental evolution in the Symbiodiniaceae and with further work into the Symbiodiniaceae-coral association and bleaching response, this approach may become a valuable tool in coral reef conservation and restoration initiatives.

4.2 Introduction

Coral reefs are the most biodiverse ecosystem in the marine world, contributing billions of dollars' worth of ecosystem services through tourism, coastal protection and fishing industries, with invaluable cultural and medicinal significance (Moberg and Folke 1999). However, reef-building corals are under serious threat from rapid ocean warming, driven by global warming, as a consequence of rising anthropogenic carbon emissions. The unprecedented rate of global change is considered too rapid for many marine organisms to keep up, leading to the prediction of mass species extinction by the end of the century (Hoegh-Guldberg and Bruno 2010; Pereira et al. 2010;

Dawson et al. 2011; Pacifici et al. 2015; Urban 2015). Increases in temperature are one of the main causes of coral bleaching, whereby the Symbiodiniaceae are lost from coral tissue in a stress response driven by the Symbiodiniaceae and/or the coral (Pandolfi et al. 2011). Corals meet most of their energy requirements from the Symbiodiniaceae living inside their cells (Falkowski et al. 1984) and although corals can survive without their photosymbionts for a limited period of time, prolonged bleaching leads to coral starvation and will ultimately lead to death. Even if corals can re-establish a symbiosis when temperatures return to normal, they may be left prone to disease (e.g. Harvell et al., 1999; Howells et al., 2016; Kushmaro et al., 1997), show reduced growth and can be reproductively compromised (e.g. Baird and Marshall, 2002; Miller et al., 2009).

Rising ocean temperatures have triggered three global mass bleaching events (1998, 2010 and 2014-2017) since records began in the 1980s. Not only have bleaching events become more extreme and widespread, but the time between successive events has declined by 4.6-fold from the early 1980s (Hughes et al., 2017), giving surviving coral less time to recover. Ocean warming is unlikely to be curbed, with models showing only a 5% chance that the global temperature increase since pre-industrial times will be less than 2 °C by 2050 (Raftery et al. 2017). To preserve just ≤10% of the world's coral reefs, it has been predicted that warming must be limited to below 1.5°C (Frieler et al. 2013). Some marine species may be able to adapt, acclimate or simply move to cooler places in response to rapid ocean warming (Cheung et al. 2009), however for reef-building corals that have relatively long generation times (4-20 years, Babcock 1991) are already near their physiological thermal limits and are sessile, such options are limited. Therefore, the need to develop measures that enhance the thermal resilience of corals is pressing.

Assisted evolution is the acceleration of naturally occurring evolutionary processes to enhance certain traits; for tropical corals, it involves a set of direct interventions and aims to increase climate resilience (van Oppen et al. 2015; van Oppen et al. 2017). One approach that has recently been pioneered is to make use of the comparatively short generation time of the asexually reproducing Symbiodiniaceae*,* by subjecting them to laboratory thermal selection. Symbiodiniaceae generation times *in hospite* correspond to between three and 74 days (Muscatine et al. 1984; Hoegh-Guldberg et al. 1987; Wilkerson et al. 1988) and may be restricted by the host through the amount of nutrients provided, host digestion and expulsion (Cantin et al., 2009; Cook et al., 1988; Muscatine and Pool, 1979; Stat et al., 2008; Titlyanovl et al., 1996), which could limit their rate of adaptation. However, in culture, typical doubling times for the Symbiodiniaceae are often less than three days (Fitt and Trench 1983; Taguchi and Kinzie III 2001; Kinzie et al. 2001), and in a laboratory

environment, the Symbiodiniaceae can be cultured *ex-hospite* under strong thermal selection conditions that encourage large population sizes and minimise generation times, thus maximising the rate of genetic adaptation (Bromham 2009). As coral survival is highly dependent on the thermal performance of their symbionts, such research may hold the key to reducing the extent of coral bleaching under thermal stress (**Chapter 2**; van Oppen et al., 2015).

The family Symbiodiniaceae comprises multiple species (LaJeunesse et al. 2018), that were formerly described as clades and types within the genus *Symbiodinium* (van Oppen et al. 2005b; Pochon and Gates 2010). Corals can host multiple species simultaneously (Boulotte et al., 2016; Mieog et al., 2007; Silverstein et al., 2012) and dominant Symbiodiniaceae species can vary in relative abundance, depending on coral life-stage (e.g. Little *et al.*, 2004) and environmental factors (e.g. Baker et al., 2004; Rowan, 2004). Most importantly, the association of specific Symbiodiniaceae species might alter the thermal stress tolerance of the holobiont (Berkelmans and van Oppen 2006a; Sampayo et al. 2008; Abrego et al. 2009; Mieog et al. 2009b; Stat and Gates 2011; Howells et al. 2012). Members of the genus *Durusdinium* (formerly ITS2 *Symbiodinium* clade D) in particular have attracted attention as they have been linked to high bleaching tolerance in corals, where under heat stress, they out-compete other, perhaps more functionally beneficial Symbiodiniaceae (reviewed by Stat et al., 2008). However, **Chapter 2** demonstrated that for a laboratory evolved strain of *Cladocopium goreaui* (formerly ITS2 type *Symbiodinium* C1) that had a widened thermal niche, there was limited transfer of heat tolerance when in symbiosis. Such responses could be strain-specific and thus it is important to examine the response of a wide taxonomic diversity of Symbiodiniaceae species to temperature selection for their potential use in assisting coral evolution.

Here, I carried out a laboratory selection experiment with five genetically different Symbiodiniaceae isolated from the Great Barrier Reef (GBR, *Symbiodinium*, two strains of *Durusdinium*, *Fugacium*, and *Gerakladium*), by exposing cultures to increasing temperatures, up to 34°C, for one year. Subsequently, I investigated the thermal tolerances of these selected Symbiodiniaceae (SS) cultures relative to the unselected wild-type (WT) cultures that had been kept under control temperature conditions of 27°C.

4.2 Materials and methods

Symbiodiniaceae isolation and culture

Five strains of the Symbiodiniaceae were isolated from four species of coral and one species of giant clam from the central and southern Great Barrier Reef (**Table 4.1**). A *Fugacium* culture and

Symbiodinium culture were obtained from the University of Technology Sydney (**Table 4.1**), and are described in (Suggett et al. 2015) while the remaining strains were isolated and cultured at the Australian Institute of Marine Science. This involved air-brushing the coral tissue, centrifuging (5 min, 1600 g), decanting, and re-suspending the pellet three times in 0.2 µm filtered seawater. Individual cells were transferred into sterile culture media, Daigo's IMK for Marine Microalgae (Nihon Pharmaceutical Co., Ltd) containing antibiotics; penicillin, neomycin, streptomycin, nystatin (final concentration 100 µg mL^{-1}), amphotericin (final concentration 2.5 µg mL^{-1}), and GeO_2 (final concentration 50 µM, Beltran et al., 2012). To minimise bacterial growth, cells were inoculated into fresh IMK+antibiotics monthly, for five months. Each culture was genotyped based on the ITS2 rDNA region and annual sequencing checks confirmed that the cultures did not become contaminated during longer-term laboratory culture. Cultures were maintained at 27°C in an environmental chamber (Steridium, er-rh-500) and 65 ± 10 µmol photons m^{-2} s^{-1} (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle. It is important to note that isolation of symbionts from hosts may not necessarily represent their dominant symbiont genera or species. Additionally, it is possible that the Symbiodiniaceae strains cultured could have either represented free-living cells, perhaps associated with the corals surface, or symbiotic cells harboured within the hosts' tissues. However, in preliminary trials, all five strains were able to infect the coral species *Acropora tenuis* and *A. millepora* suggesting that they are true symbionts (LJC, unpubl.).

Table 4.1. Symbiont genera, ITS2 type and site of origin for each Symbiodiniaceae strain used in this study.

Strain Identification	Genera	ITS2 type	Host species	Geographic Origin	GenBank Accession number
CS73/SCF022.01	*Symbiodinium*	A3c	*Tridacna maxima* (giant clam)	Heron Island (southern GBR)	KR013750
SCF082	*Durusdinium*	D1	*Acropora muricate* (coral)	Magnetic Island (central GBR)	MH229352
SCF086.01	*Durusdinium*	D1	*Porites lobata* (coral)	Davies Reef (central GBR)	MH229353
UTSC/SCF089.01	*Fugacium*	F1	*Pocillopora damicornis* (coral)	Heron Island (southern GBR)	AF427462
SCF097.01	*Gerakladium*	G3	*Diploastrea heliopore* (coral)	Davies Reef (central GBR)	MH229354

Symbiodiniaceae thermal selection experiment

Experimental design

A thermal selection experiment was carried out using a modified ratchet design (Huertas et al. 2011, **Figure 4.1**). The ratchet design is a method used to maximise the number of spontaneous mutations

arising from asexual cell division, by maintaining large population sizes while increasing levels of thermal selection pressure in a step-wise fashion.

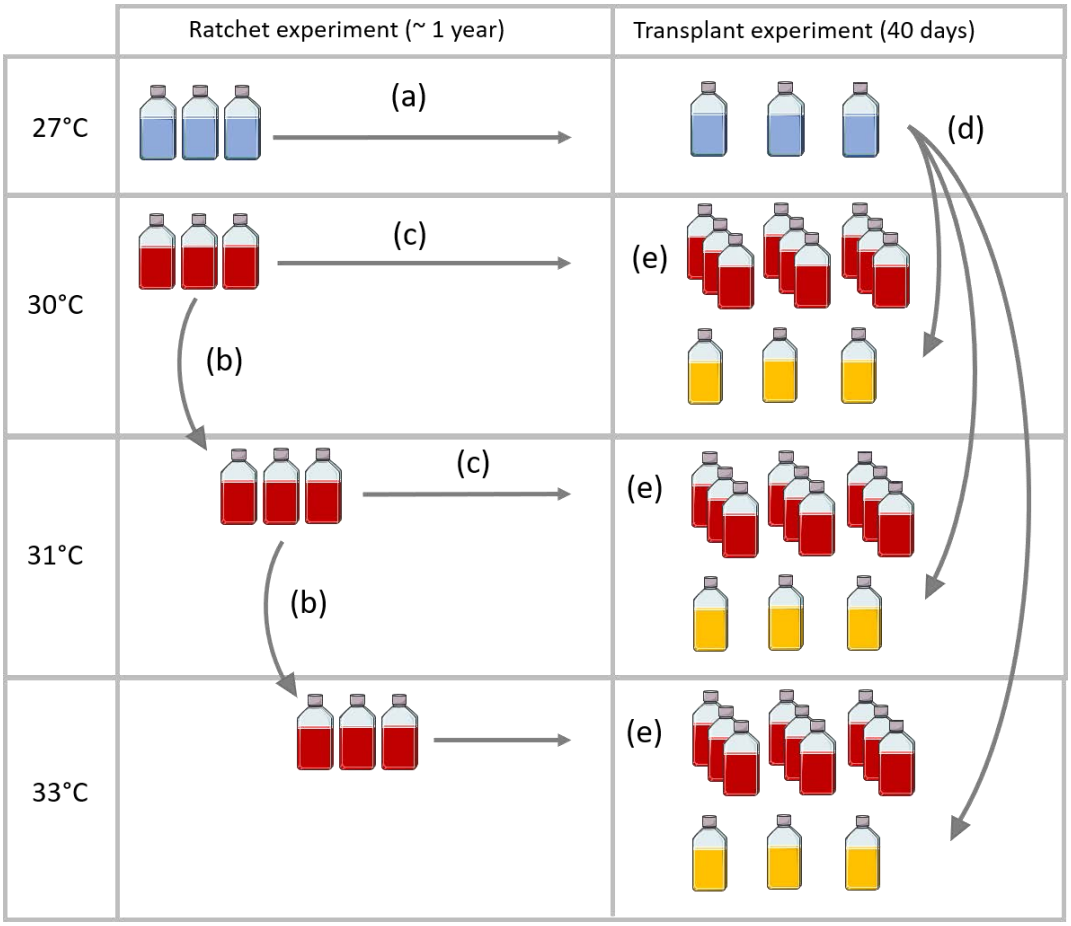

Figure 4.1. Simplified experimental design to show long-term thermal selection ending in an acute heat stress transplantation experiment. (A) Control replicate cultures were kept at 27°C throughout the duration of the long-term selection experiment. (B) replicate populations were moved to the next, elevated temperature treatment if after 6 weeks they exhibited positive growth. (C) For populations that were eligible to be transferred to the next temperature treatment, an aliquot was also kept at the same temperature conditions throughout the experiment. (D) After one year, control replicate cultures were transferred to each of the elevated temperature treatments that the temperature-selected populations had achieved (E) after one year of thermal selection, thermally selected populations were split into three replicate cultures and maintained in their own temperature conditions. A 40-day transplant experiment commenced.

The ratchet experiment involved five temperature treatments starting at control temperature conditions of 27°C (27.04°C ±0.002, n=54275) and 30°C (30.06°C ±0.0008, n=54347), with further 1-2°C increments; 31°C (31.17°C ±0.002, n=48123), 33°C (32.78°C ±0.006, n=35702) and 34°C (34.04°C ±0.001, n=14045), under the same light conditions as their prior culture (65 ± 10 µmol photons m^{-2} s^{-1}, 14:10 light:dark cycle) and in five temperature-controlled environmental chambers (Steridium, er-rh-500). Temperature measurements were recorded every 10 min, using a data logger (HOBO Pendant®). To begin the ratchet experiment, replicate control cultures (n = 3) of each strain were

inoculated into fresh media at 100,000 cells mL^{-1} to give a final volume of 20 mL of culture and kept at 27°C (control) and the first treatment temperature of 30°C (**Figure 4.1**). This jump in temperature from 27°C to 30°C was chosen based on previous observations showing most Symbiodiniaceae can survive 30°C. Importantly, once the experiment started, each 'replicate' culture at 30°C (or subsequent temperature treatments) was no longer considered a replicate but an independent evolutionary unit in which different random mutations could arise (Huertas et al. 2011). The first ratchet level consisted of populations' (both control and treatment) being cultured for a three-week pre-acclimation period before being inoculated into fresh media at the same starting concentration of 100,000 cells mL^{-1} and being cultured for a further three weeks before being transferred to fresh media again. Every three weeks, aliquots of each population were taken to determine the growth rate, which was used to calculate the number of generations each replicate had gone through (see 3.3.2 for calculation of growth rate number of generations).

At the end of the six-week ratchet, if an individual population at 30°C showed positive growth, the population was transferred into the next temperature treatment (31°C), into fresh media at 100,000 cells mL^{-1}. If a population did not display positive growth it remained at the same temperature until the next ratchet cycle when growth performance was assessed again. The experiment continued in this way, with populations being transferred to incremental temperature increases every 6 weeks, where appropriate (Fig. 1).

My ratchet experimental design differed from that of Huertas et al. (2011) in three ways. Firstly, I allowed six weeks before assessing the growth of each population and deciding to move a population(s), whereas microalgal growth was assessed after only 15-20 days in Huertas et al. (2011). Six weeks at each treatment temperature was important in my experiment because I observed that for one strain (type D1, SCF086) the full, negative effects of elevated temperature on growth were cumulative and not observed before three weeks, which may have led to an uninformed decision on which populations were suitable to transfer to the next ratchet temperature. Alternatively, I did not want to mistake an initial, positive plastic response for a stable adaptive change. Secondly, I transferred cells into the next selecting temperature if they exhibited any positive growth, whereas in Huertas et al. (2011) only if a population exhibited growth equal to, or greater than, the control population, was it transferred; my decision was made based on **Chapter 2** where a laboratory selected SS strain of *Cladocopium goreaui* that had been cultured for approximately 80 generations at 31°C was not able to match the growth rate of its WT counterpart at the control temperature, but exceeded the growth of the WT transferred short-term into elevated

temperature. Thirdly, even if a population was transferred to the next temperature treatment, a replicate was also always maintained at the previous temperature treatment(s). Previous temperatures may have been sub-optimal for a population, even if they were able to maintain some growth, thus more generations of culture could have allowed a (further) adaptive response to occur.

Testing for a stable adaptive response

To test whether a stable adaptive response had occurred after ~ 1 year of thermal selection in the ratchet experiment, I carried out a transplantation experiment. This involved control or wild-type (WT) populations being both kept at 27°C (WT@27, 26.84°C ±0.005, n = 4394) and also transferred to the elevated temperature treatments e.g. WT@30 (29.89°C ±0.0004, n=54347), WT@31 (31.02°C ±0.021, n=4322), WT@33 (32.93°C ±0.016, n=4409) and/or WT@34 (34.08°C ±0.0009, n=4322). Growth rates and photophysiological measurements of the selected Symbiodiniaceae populations (SS) already at elevated temperature (SS@30, SS@31 etc.) were compared to those from WT cultures at control temperature moved to elevated temperatures.

Each WT population was transferred to fresh media at a cell density of 200,000 cells mL^{-1} and pre-acclimated for two weeks (up to nine generations) to each temperature treatment that their counterpart SS resided in. SS populations were also transferred to fresh media and cultured for two weeks at their appropriate temperature conditions. The pre-acclimated cells were then transferred into fresh media at the same starting concentration to give three replicate cultures (n=3) for each population in each temperature treatment.

On days 3, 10, 17 and 26 after inoculation, 100 µL (0.5% of total volume) aliquots of homogenised culture from each WT and SS replicate was transferred into a black, clear-bottom 96-well culture plate (Costar, Corning; Sigma-Aldrich) for photophysiology and growth rate measurements.

Photophysiology

Culture aliquots were transferred to multi-well plates during the end of the dark period and were dark-adapted for a minimum of a further 30 mins at their appropriate temperature treatments. The maximum quantum yield of PSII ($F_v/F_m = (F_m - F_o)/F_m$) was measured on dark-adapted samples using an imaging PAM (iPAM, Walz, Germany) with the measuring light set at 10 and gain and damping at two. The cultures were then subjected to six minutes of actinic light of two (PAR=20 µmol photons $m^{-2}\ s^{-1}$) before measuring the effective quantum yield of PSII ($\Delta F/F_m' = ((F_m' - F')/ F_m')$. Cells were

then fixed in the well-plates by adding 3.4 µL of 25% glutaraldehyde for later cell counts and calculation of growth rate.

Growth rate and number of cell generations

To measure growth rate, cell density was determined on day 17 post-acclimation in each replicate by triplicate haemocytometer counts. The specific growth rate (µ, doubling/day) was calculated as

$$\mu = \frac{lnN_1 - lnN_0}{\Delta t}$$

where N_0 is the cell density at the start (200,000 cells/mL) of the experiment and N_1 is the cell density at day 17 and *t* is the duration of culture (17) in days. To estimate the number of generations each population had been through during long-term selection, the doubling time (or generation time) was calculated, according to the equation

$$T = d\ x\ (\frac{\log(2)}{\log(\frac{N_1}{N_0})})$$

Where *d* is the number of days of growth and N_0 is the initial inoculation cell density (i.e. 100,000 cells mL^{-1} during the ratchet experiment and 200,000 cells mL^{-1} during the transplant experiment). Subsequently, the number of generations was calculated as

$$Generations = N/T$$

Where *N* is the number of days of culture and *T* is the doubling time.

Statistical analyses

To test whether the selected Symbiodiniaceae (SS) populations had mounted an adaptive response as a result of long-term thermal selection, I compared the growth and photosynthetic performance of the three SS populations (a-c) at each temperature treatment that they had reached (e.g. SS@30a/b/c, 31, 33 and/or 34) with the performance of the control population, maintained at 27°C long-term (e.g. WT@27) and the WT population transferred into the elevated temperature treatment (e.g. WT@30, WT@31, WT@33 and/or WT@34). This was done for each Symbiodiniaceae strain using linear models with "population" (i.e. WT@27, WT@30, SS@30a, SS@30b, SS@30c) as a fixed factor for both growth rate and photosynthetic efficiencies and with "time" as an additional fixed factor for the latter. Models were carried out using the package 'nlme' (Pinheiro et al. 2017). All analyses were conducted in R v. 3.4.1 (R Core Team 2016).

4.3 Results

Long-term culture

One strain of *Durusdinium* (SCF082), was not able to grow at the first ratchet temperature of 30°C, despite two, 6-week attempts of culture and was therefore could not be included in the subsequent transplant experiment. The *Symbiodinium,* other *Durusdinium* (SCF086.01), *Fucacium* and *Gerakladium* populations all exhibited positive growth at 30°C and were cultured for between 25 and 71 generations compared with 47-73 generations at 27°C (**Table 4.2**). Only two *Fucacium* and *Gerakladium* populations, were able to grow at the next ratchet temperature of 31°C with all three SS populations of both types cultured at 31°C for up to 69 and 53 generations, respectively. At 33°C and 34°C only *Fugacium* populations weres able to grow, with the three SS populations having gone through 41-46 generations at this temperature by the end of the ratchet experiment. At the highest ratchet temperature, *Fucacium* was cultured for between 16 and 17 generations, before the transplant experiment was carried out.

Table 4.2. Number of generations that each population spent at each temperature condition during the ~1-year ratchet experiment.

		Generations at each temperature					
Symbiodiniaceae ITS2 type	Population	27°C	30°C	31°C	33°C	34°C	Cumulative generations
A3c	WT@27a	50					50
	WT@27b	52					52
	WT@27c	51					51
A3c	SS@30a		46				46
	SS@30b		44				44
	SS@30c		41				41
D1	WT@27	47					47
(SCF086.01)	WT@27	48					48
	WT@27	49					49
D1	SS@30a		26				26
(SCF086.01)	SS@30b		26				26
	SS@30c		25				25
F1	WT@27	69					69
	WT@27	72					72
	WT@27	70					70
F1	SS@30a		69				69
	SS@30b		66				66
	SS@30c		67				67
F1	SS@31a		9	62			70
	SS@31b		9	59			68
	SS@31c		9	61			70
F1	SS@33a		9	18	41		68
	SS@33b		9	18	42		69
	SS@33c		9	18	43		70
F1	SS@34a		9	18	26	17	69
	SS@34b		9	18	26	16	69

	SS@34c		9	18	25	17	68
G3	WT@27a	73					73
	WT@27b	75					75
	WT@27c	71					71
G3	SS@30a		71				71
	SS@30b		69				69
	SS@30c		70				70
G3	SS@31a		6	52			58
	SS@31b		6	52			57
	SS@31c		6	51			57

Testing for a stable adaptive response (transplantation experiment)

***Symbiodinium* (A3c)**

Two of the *Symbiodinium* SS populations (SS@30a and SS@30c) were able to grow significantly faster than the WT@30 population (max t_2=5.28, P<0.01, **Appendix 4.1**) by up to 49% (±8.80%) after long-term culture at 30°C, with the growth rate of population SS@30a being significantly faster than the WT@27 (t_2=3.51, P=0.04, **Appendix 4.1**), by 28% (±7.53% **Figure 4.2A**). Mean effective photosyntheticyields for the three SS@30 populations, were between 10.68% (±1.45%) and 21.75% (±2.61%) lower than the controls throughout the experiment, however they remained stable over the 26 days period (min t = -1.79, P=0.71, **Figure 4.2B**). In contrast, the WT@30 population, showed a significant decline of 17.19% (±4.75%) in mean effective photosynthetic quantum yield by the end of the experiment with a final mean value that was significantly lower than that of the three SS@30 populations (max t =5.56, P<0.01, **Appendix 4.1**) by up to 23.92% (±4.75%, **Figure 4.2B**).The maximum quantum yield values followed a similar trend (**Figure 4.2C**), although the drop in WT@30 mean yield value by the end of the experiment was not as marked.

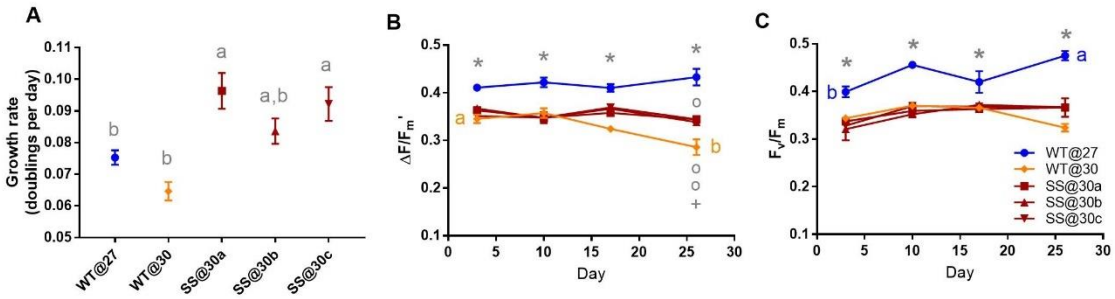

Figure 4.2. *Symbiodinium* (ITS2 type A3c). Results of transplant experiment; comparison of mean (±SEM) growth rates (A), effective quantum yields (B) and maximum quantum yields (C) between the wild-type (WT) A3c population, WT transferred into 30°C (WT@30) population and the three selected A3c populations (SS@30a, b, c). Populations' were pre-acclimated for 14 days and growth rates were measured following 17 more days and quantum yield measurements over the following 26 days. Statistically significant differences among populations at a given time point are represented by different letters (A) or symbols above/below the mean points (B, C). Statistically significant differences over time (between D3 and D26) are represented by

different letters (B and C, Tukey's post hoc tests; P < .05), n = 3 for all means. Where error bars are not visible, they are small and hidden by the symbols

Durusdinium (D1)

Long-term culture at 30°C did not improve the mean growth of any D1 SS population. In fact, one population, SS@30b grew 43.8% (±15.2%) slower than the WT@30 (t_2=-3.84, P=0.02, **Figure 4.3A**, **Appendix 4.2**). There was some variation in growth rates among the three SS populations, with SS@30b growing 43.1% (±15.4%) and significantly slower than SS@30c (t_2=3.73, P=0.03, **Appendix 4.2**). In general, the SS@30 populations displayed a trend of both lower effective quantum yield values and maximum quantum yield values compared to both the WT@27 and WT@30 populations during the transplant experiment (**Figures 4.3B, C**), despite long-term thermal exposure at 30°C.

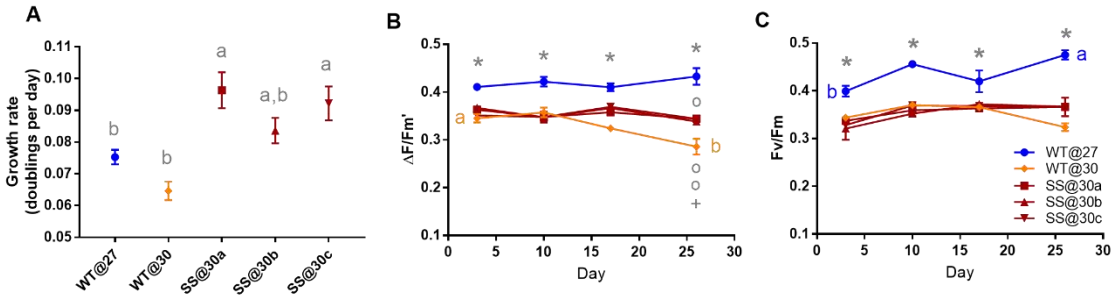

Figure 4.3. *Durusdinium* (ITS2 type D1). Results of transplant experiment; comparison of mean (±SEM) growth rates (A), effective quantum yields (B) and maximum quantum yields (C) between the wild-type (WT) D1 population, WT transferred into 30°C (WT@30) and the three selected D1 populations (SS@30a, b, c). Populations' were pre-acclimated for 14 days and growth rates were measured following 17 more days and quantum yield measurements over the following 26 days. Statistically significant differences among populations at a given time point are represented by different letters (A) or symbols above/below the mean points (B, C). Statistically significant differences over time (between D3 and D26) are represented by different letters (B and C, Tukey's post hoc tests; P < .05), n = 3 for all means. Where error bars are not visible, they are small and hidden by the symbols

Fugacium (F1)

After long-term thermal selection, one population, SS@30b, grew 24.84% (±0.95%) slower compared to the WT@27 (t_2=-3.810, P= 0.02, **Appendix 4.3**) and 23.80% (±0.96%) slower compared to the WT@30 (t_2=-3.60, P=0.03, **Figure 4A**, **Appendix 4.3**), while the remaining two SS@30 populations (a and c), maintained mean growth rates that were similar to the WT@27 and WT@30 (min t_2=-2.37, P=0.20) . By day 17 and 26, WT@27 and WT@30 effective quantum yield values were reduced by as much as 24.74% (±1.25%), compared to all three SS populations, which had statistically similar yields (min. t=-1.62, P=0.98, **Figure 4B**). Similarly, maximum quantum yield values became reduced for the WT@27 and WT@30 populations at day 26; by up to 19.56% (±0.40%) compared with the SS populations (±0.14%, min t=-8.12, P<0.01, **Figure 4C**, **Appendix 4.3**).

Long-term culture at 31°C did not significantly affect the growth rates of the F1 SS populations nor did the short-term transfer of WT cells into 31°C (WT@31, max t_2=2.73, P=0.12, **Figure 4D**). During the latter half of the experiment, the three SS@31 populations and the WT@31 population displayed mean effective quantum yield values that were up to 17.26% (±1.93%) greater than the mean values for the WT@27 control (max t=5.90, P<0.01, **Figure 4E**, **Appendix 4.4**). Similar trends were apparent for the mean maximum quantum yield values (**Figure 4F**), with the WT@27 having significantly lower values than the remaining treatments by up to 13.50% (±0.94%) at day 26 (max t =5.98, P<0.01, **Appendix 4.4**).

Similarly to the 31°C experiment, no significant differences in growth rate existed between any F1 population in the 33°C transplant experiment (max t_2=2.00, P=0.33, **Figure 4G**). Furthermore, differences in both mean effective quantum yields and maximum quantum yields by the end of the experiment, were minimal (**Figures 4.4H, I**, **Appendix 4.5**).

After long-term selection for F1 populations at 34°C, there were no significant differences in mean growth rates between the three populations (max t=-1.65, P=0.50), however, the SS@34b population exhibited a mean growth rate that was 22% (±2.10%) faster than the WT@34 population (t_2=3.81, P=0.02, **Figure 4.4J**, **Appendix 4.6**). Effective quantum yield values between all F1@34 populations, by the end of the experiment, were similar (max t= 0.20, P = 1.00, **Figure 4.4K**). Further, maximum quantum yield values were not significantly different to each other between the populations by the end of the experiment (max t= -2.14, P = 0.82 **Figure 4.4L**).

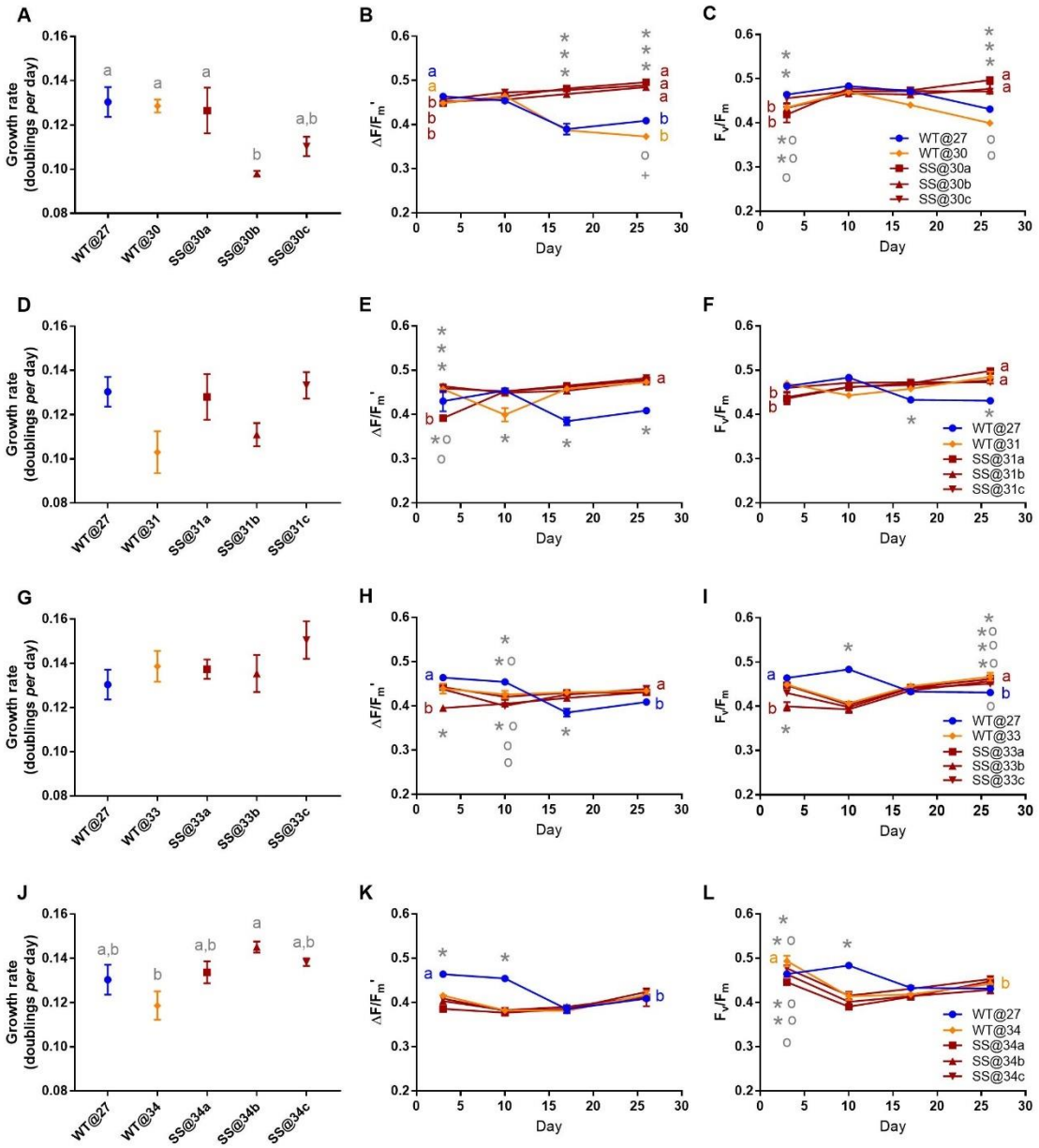

Figure 4.4. *Fugacium* (ITS2 type F1). Results of transplant experiment; comparison of mean (±SEM) growth rates (A, D, G, J), effective quantum yields (B, E, H, K) and maximum quantum yields (C, F, I, L) between the wild-type (WT) F1 population, WT transferred into elevated temperature (WT@30, WT@31, WT@33 or WT@34) and the three selected F1 populations (e.g. SS@34a, b, c). Populations' were pre-acclimated for 14 days and growth rates were measured following 17 more days and quantum yield measurements over the following 26 days. Statistically significant differences among populations at a given time point are represented by different letters (A) or symbols above/below the mean points (B, C). Statistically significant differences over time (between D3 and D26) are represented by different letters (B and C, Tukey's post hoc tests; $P < .05$), $n = 3$ for all means. Where error bars are not visible, they are small and hidden by the symbols.

***Gerakladium* (G3)**

There were no significant differences in growth rate between any of the G3 populations in the 30°C transplant experiment (max t_2=1.71, P=0.47, **Figure 4.5A**). There were also no notable differences in effective quantum yield values by the end of the experiment (max t_2=0.03, P=1.00, **Figure 4.5B**, **Appendix 4.7**) or maximum quantum yield values (max t_2=1.445, P=0.10, **Figure 4.5C**, **Appendix 4.7**).

Wild-type (WT) G3 cells transferred into 31°C grew 27.9% (±1.78%) slower, compared to the WT@27 controls (t_2=-4.23, P=0.01, **Appendix 4.8**). For two of the SS populations (b and c) growth rates were intermediate to that of WT@31 and WT@27, and not significantly different (min t_2=-2.26, P=0.24). However, for the remaining SS@31a population, the mean growth rate was similar to the WT@27 cells (t_2=-2.260, P=0.999) and significantly greater, by 36% (±9.82%), than the WT@31 cells (t_2=3.97, P=0.02, **Figure 4.5D**, **Appendix 4.8**). The mean effective quantum yield for the WT@31 population was significantly lower by up to 13.47% (±3.74%) compared to the WT@27, SS@31a and SS@31b populations at 3 days post-acclimation (max t = -3.92, P=0.04, **Appendix 4.8**). By the end of the experiment, however, mean effective quantum yields were similar among all populations (min t=-0.57, P = 1.00, **Figure 4.5E**). Again, at 3 days post-acclimation the WT@31 population had the lowest maximum quantum yield values compared to the WT@27, SS@31a and SS@31b populations (max t = -4.06, P=0.02, **Figure 4.5F**, **Appendix 4.8**), by up to 17.27% (±4.62%), but by the end of the mean values were similar among all populations (min. t -0.57=, P=1.00, **Figure 4.5F**).

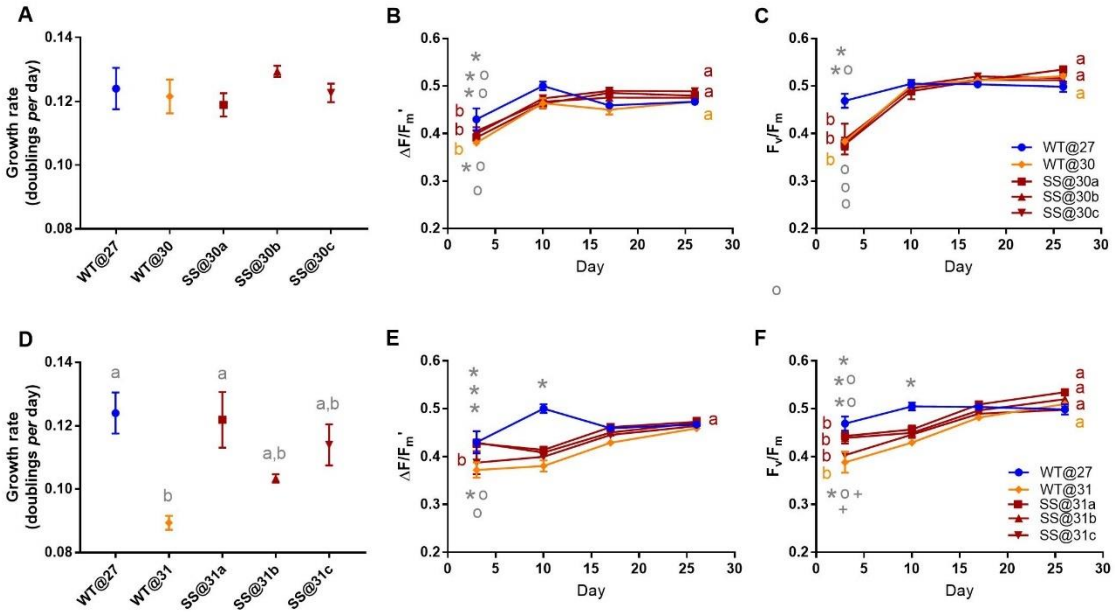

Figure 4.5. *Gerakladium* (ITS2 type G3). Results of transplant experiment; comparison of mean (±SEM) growth rates (A, D), effective quantum yields (B, E) and maximum quantum yields (C, F) between the wild-type (WT)

G3 population, WT transferred into 30°C or 31°C (WT@30 or WT@31) and the three selected G3 populations (e.g. SS@30a, b, c). Populations' were pre-acclimated for 14 days and growth rates were measured following 17 more days and quantum yield measurements over the following 26 days. Statistically significant differences among populations at a given time point are represented by different letters (A) or symbols above/below the mean points (B, C). Statistically significant differences over time (between D3 and D26) are represented by different letters (B and C, Tukey's post hoc tests; $P < .05$), $n = 3$ for all means. Where error bars are not visible, they are small and hidden by the symbols

4.4 Discussion

The thermal tolerance response to long-term exposure to increasing temperatures of five Symbiodiniaceae wild-types (WT) representing four genera exhibited considerable variation. After one year of culture at step-wise increases in temperature, three of the five Symbiodiniaceae showed signs of a stable adaptive change; two thermally-derived populations of type *Symbiodinium* (ITS2 type A3c) at 30°C, one of *Gerakladium* (ITS2 type G3) at 31°C and one of *Fugacium* (ITS2 type F1) at 34°C after only 41-69 generations at elevated temperature showed increased performance at elevated temperatures compared to the respective WT cultures.

Symbiodinium (A3c)

The acute heat stress (i.e., transplant) experiment of the WT A3c cells into the first elevated temperature treatment of 30°C resulted in a 17% decrease in growth rate and declines in photosynthetic efficiencies, suggesting a relatively low natural thermal tolerance for this strain. This is perhaps surprising as the literature describes most *Symbiodinium* (formerly clade A) strains as thermally tolerant (Kemp et al., 2014; Reynolds et al., 2008; Robison and Warner, 2006) and typically prevalent in shallow-water cnidarians, where high light and temperature co-occur (LaJeunesse 2002). This has generally been attributed to enhanced and alternative photosynthetic electron pathways such as cyclic electron transport and light harvesting complex dissociation mechanisms as well as photoprotection processes (Reynolds et al. 2008). In contrast, two of the SS A3c populations in my study that were cultured for up to 46 generations at 30°C, were able to grow 43-49% faster than the WT@30 and up to 28% faster than the WT at the control temperature. Furthermore, throughout the transplant experiment, the photosynthetic efficiencies of the WT@30 significantly decreased over time, while the SS populations were able to maintain high photosynthetic efficiencies. These results suggest a stable, adaptive change having occurred after only up to 46 generations at 30°C.

Current evidence on the value of members of *Symbiodinium* to the coral host is varied. *Symbiodinium* have been associated with the fast colonisation of health-compromised corals (Toller et al. 2001), low transfer of photosynthate compared to *Cladocopium* (ITS2 type C1) (Stat et al.,

2008) and high competitive ability in mixed *in vitro* cultures (Rowan 1998). Furthermore, there have been reports of wide phenotypic and physiological diversity of *Symbiodinium* (Krämer et al., 2012; Ragni et al., 2010; Robison and Warner, 2006; Takahashi et al., 2009). These factors have led to the suggestion that members of *Symbiodinium* are more parasitic in nature and suboptimal as symbionts (Mieog et al., 2009; Stat et al., 2008). However, other studies have suggested their ecological value*; Symbiodinium* (ITS2 type A3c) was associated with juvenile GBR *Acropora tenuis* that had lower mortality than when harbouring other types of Symbiodiniaceae (Quigley et al. 2016) and early larval uptake of *Symbiodinium* in *Acropora yongei* was associated with a higher post-settlement survival rate (Suzuki et al. 2013). These studies suggest that some members of *Symbiodinium* could be valuable in experimental evolution studies aimed at enhancing the tolerances of the early life stages of coral. Thus, the stable enhanced thermal tolerance I observed here is promising, particularly because I observed this in two of three SS populations.

***Durusdinium* (D1)**

I experimented with two *Durusdinium* (ITS2 type D1) strains of Symbiodiniaceae that are genetically distinct based on ITS2 sequences. Surprisingly, neither were particularly thermally tolerant, considering members of *Durusdinium* are widely described as being heat resistant in the literature (Glynn et al. 2001; Toller et al. 2001; Pettay et al. 2015). The D1 strains, SCF082 and SCF086.01, were not able to grow at, or above 30°C, respectively; for D1 (SCF086.01), SS populations managed to grow for only 25-26 generations at 30°C over a 1-yr period, compared to the 47-49 generations of the WT@27. Furthermore, long-term culture at 30°C had a negative effect on the photosynthetic efficiencies of the SS populations and a 44% decrease in growth of one population. In contrast, *Durusdinium* has often been found to dominate corals on reefs that are exposed to heat stress or have had a history of bleaching (Glynn et al. 2001; Toller et al. 2001; Rowan 2004; Baker et al. 2004; van Oppen et al. 2005b; Jones et al. 2008; LaJeunesse et al. 2009; Pettay et al. 2015) and has been shown to enhance the thermal tolerance of the coral *Acropora millepora,* a common Indo-Pacific species, by 1-1.5°C through the shuffling of symbiont types (Berkelmans and van Oppen 2006a). Other studies have shown high abundances of *Durusdinium* in corals at 32°C in Thailand (LaJeunesse et al. 2010), 31.8°C in Palau (Fabricius et al. 2004) and >33°C in the Persian Gulf (Baker et al. 2004; Mostafavi et al. 2007). This suggests that there is either wide variation in thermal tolerance within the genus *Durusdinium* and/or thermal tolerances are different in free-living cultures compared to *in hospite* populations.

***Fucagium* (F1)**

Fugacium (ITS2 type F1) was the most thermally tolerant strain in this study in terms of its ability to mount a plastic response. In the transplant experiments, growth rates of the WT transferred to 30, 31, 33 and 34°C did not significantly change, while photosynthetic efficiencies remained similar. This agrees with previous *in vitro* studies on other members in this clade; between 25°C and 33°C, a different strain of F1 did not show any differences in growth or photosynthetic efficiencies over two weeks, while expressing significantly higher levels of antioxidant defence enzymes at 33°C aimed to scavenge reactive oxygen species (ROS) produced as a by-product of photosynthesis and cellular respiration (Krueger et al. 2014). The ability to mitigate damage caused by excess ROS was also shown by an ITS2 type F2 population after 12 days at 29-31°C (McGinty et al., 2012), while the same F2 isolate showed the greatest capacity for both photo-acclimation and growth at 32°C compared with isolates belonging to clades A and B (Robison and Warner, 2006).

Long-term culture in this study did not significantly affect the ability of F1 to grow or its photosynthetic efficiency under elevated temperature, apart from two cases. Firstly, 66-67 generations at 30°C resulted in a significant drop in growth rate for two SS populations (SS@30b and c), with SS@30b being statistically lower than the WT@30 transplant by 23.80% and WT@27 control by 24.84%. Despite this, the photosynthetic efficiencies of all three SS populations were greater than the WT@27 and WT@30 across much of the transplant experiment and remained stable. Secondly, long-term culture at 34°C enhanced the growth rate of one SS population (SS@34b) such that it grew 18% faster than the WT@34, suggesting some temperature evolution

Members or *Fugacium* are rarely described in the literature, in symbiosis or as free-living populations. *Fugacium* made up only 0.9% of Symbiodiniaceae metagenomic sequences recovered from ten coral species, sediments and water samples across contrasting thermal environments in American Samoa (Cunning et al. 2015b). In the Indian Ocean, 47 ITS2 Symbiodiniaceae types were characterised from over 600 samples only one was found to harbour a member of *Fucgacium*, an anemone species (LaJeunesse et al. 2010). Only 1 of 16 coral species, *Goniastrea* sp., around the Arabian Peninsula harboured *Fugacium* (ITS2 type F4.3) and at >5% abundance (Ziegler et al. 2017). In the GBR, eastern Pacific and Caribbean 114 coral species revealed no *Fugacium* (van Oppen et al. 2005a). Such a lack of biogeographic prevalence and abundance is surprising given this clade's ability to cope with heat stress. This calls for the need to study the efficacy of *Fugacium* as a symbiont.

***Gerakladium* (G3)**

After 57 generations at 31°C, a population of *Gerakladium* (ITS2 type G3) was able to grow 36% faster than the WT@31, with small but apparent improvements in photosynthetic efficiencies. These results suggest a stable adaptive change had occurred after only one year at elevated temperature for at least one of the SS G3 populations.

Gerakladium has been found to associate with a wide variety of hosts and environments including sponges (Schönberg and Loh 2005; Schönberg et al. 2008; Granados et al. 2008; Hill et al. 2011), soft corals (van Oppen et al. 2005b) and foraminiferans (Pochon et al. 2001), and is also known to occur in the water column and in benthic sediments (Takabayashi et al. 2012). Occasionally, members of *Gerakladium* have been found to associate with scleractinian corals including *Porites lobata* in Hawaii (Stat et al. 2013) and low frequencies (>0.09% of all sequences) have been observed in *Orbicella annularis* in US Virgin Islands (Edmunds et al. 2014; Pochon et al. 2014) and *Acropora* in Western Australia (Thomas et al. 2014).

Although members in this genus do not seem to be common symbionts of reef-building corals, my study strain, extracted from a scleractinian coral species *Diploastrea heliopora* in the GBR, adds to the growing body of evidence of *Gerakladium* being able to form a symbiosis with scleractinian corals. Its ability to exhibit a stable adaptive change after one year of culture at 31°C, is promising for evolutionary experiments aimed to develop heat resistant Symbiodiniaceae.

Experimental evolution in microalgae and mechanisms underlying adaptive changes

To my knowledge, there have been five experimental evolution studies involving other species of marine microalgae that have subjected cells to long-term elevated temperature (**Table 4.3**). Growth rate is used as the universal indicator of fitness, allowing for comparisons between experiments. These studies indicate that after 20-460 generations (from 45 days to two years) there have been stable increases in growth of between 16% to 60% for elevated-temperature-derived strains compared to ancestral populations placed at acute elevated temperature; results comparable with my findings of increases in growth rate of 22- 49%. Evolutionary experiments involving the Symbiodiniaceae have been carried out on only three different strains, which are reported in two studies (**Table 4.3**). After only 55 generations (Huertas et al. 2011) to 80 generations (**Chapter 2**) Symbiodiniaceae were able to grow at elevated temperatures of 30°C and 31°C, respectively, where previously there had been no growth at these temperatures.

Microalgal species	Taxa	Selective condition(s)	Temperature increase	Growth rate increase	Approx. generations	Approx. duration	Study
Procentrum triestinum	dinoflagellate	25°C + elevated nutrients	5°C	12%	400	2 years	Flores-Moya et al., 2008
Isochrysis galbana	Haptophyte	35°C	5°C	*	40-50	unknown	Huertas et al., 2011
Tetraselmis suecica	Green alga	35°C	5°C	*	90-120	unknown	Huertas et al., 2011
Procentrum triestinum	Dinoflagellate	30°C	8°C	*	25-30	unknown	Huertas et al., 2011
Nitzschia closterium	Diatom	30°C	8°C	*	20-30	unknown	Huertas et al., 2011
Navicula sp.	Diatom	30°C	8°C	*	20-34	unknown	Huertas et al., 2011
Symbiodiniaceae sp.	dinoflagellate	30°C	8°C	*	55-65	unknown	Huertas et al., 2011
Symbiodiniaceae sp.	dinoflagellate	30°C	8°C	*	60-70	unknown	Huertas et al., 2011
Procentrum triestinum	dinoflagellate	25°C + reduced pH	5°C	50-60%	250	2 years	Flores-Moya et al., 2012
Emiliania huxleyi	coccolithophore	26.3°C + elevated CO_2	11.3°C	16%	460	1 year	Schlüter et al., 2014
Cladocopium goreaui	dinoflagellate	31°C	4°C	*	80	2.5 years	Chapter 2
Symbiodinium sp.	dinoflagellate	30°C	3°C	43-49%	41-46	1 year	This chapter
Fugacium sp.	dinoflagellate	34°C	7°C	22%	69	1 year	This chapter
Gerakladium sp.	dinoflagellate	31°C	4°C	36%	58	1 year	This chapter

Table 4.3. Experimental evolution studies on marine microalgae that show an adaptive response to elevated temperature. "Temperature increase" represents the increase in temperature from experimental ambient to derived experimental conditions. "Growth rate increase" is that of derived cells compared to the growth of ancestral cells transferred to derived conditions. *For some strains there was no growth when ancestral cells were transplanted into derived conditions. Shaded are experimental evolution studies on the Symbiodiniaceae.

The current study adds three more strains of Symbiodiniaceae that provide evidence of a stable adaptive change to increased temperature. These stable, physiological improvements could have resulted from either genetic adaptation through selection on random, beneficial mutations and/or acclimatisation processes that can be heritable across generations, *via* epigenetic modifications of gene expression via, for example, DNA methylation, small RNAs and chromatin modifications (Bird 2002; Greer and Shi 2012; Castel and Martienssen 2013). Under experimental heat stress a warmer water population of *Cladocopium goreaui* significantly up-regulated ROS scavenging and molecular chaperone gene expression, vital for repairing damaged proteins, compared to a cooler water population of *C. goreaui*, despite both being identical in ITS1 and ITS2 sequences and cultured under 'ambient' conditions for four years prior to experiments (Levin et al. 2016). Gene expression

responses such as these can result from genetic adaptation or epigenetic processes where historical stress induced modifications that are inherited across generations (Bonduriansky et al. 2012); either (or both) could be mechanisms for observed enhanced thermal tolerances in my study. It is possible that the historical, laboratory conditions, particularly the comparatively low light conditions that the strains were cultured under could have had some influence on their physiological responses to elevated temperature.

It is important to note that most long-term evolutionary studies on microalgae have inoculated experimental replicates with a single cell to ensure each replicate started with only one genotype (e.g Huertas et al. 2011, Lohbeck et al. 2012). In my study, cultures A3c and F1 originated from a single cell, however G3 and clades D did not. It is therefore possible that either selection on standing genetic variation and/or selection on random, beneficial mutations could have occurred in my ratchet experiment. Additionally, it is possible that for those originally monoclonal isolates, genetic diversity arising from random somatic mutations before carrying out my study may have been enough genetic variation for selection to act upon when my experiment commenced. Furthermore, my study and other evolutionary experiments on microalgae assume mitotic, asexual cell divisions produce genetic adaptations or induce heritable acclimatisation responses across generations. However, the discovery of meiosis-specific genes in the Symbiodiniaceae genome indicates that sexual reproduction is possible in the Symbiodiniaceae (Chi et al. 2014; Rosic et al. 2015; Levin et al. 2016). This has led to the hypothesis that a switch from asexual to sexual reproduction (i.e., inducing recombination) may be a response mechanism to environmental stress in the Symbiodiniaceae (Chi et al. 2014; Wilkinson et al. 2015), although it has not yet been observed and I did not observe any sexual resting stages or gametes during microscopic counts

Considerations for Symbiodiniaceae experimental evolution as a tool for coral assisted evolution

I show that *Fugacium* (ITS2 type F1) and *Gerakladium* (ITS2 type G3) are the most naturally thermally tolerant strains in this study, able to survive and grow at 34 and 31°C, respectively, even without long-term thermal selection. Despite their heat resistance, members of *Fugacium* and *Gerakladium* are some of the least prevalent in corals, with genera *Symbiodinium, Breviolum, Cladocopium* and *Durusdinium* most commonly described as being associated with scleractinians (van Oppen et al., 2009). Populations of *Durusdinium* are the most prevalent in corals after bleaching events but were the least thermally tolerant strains in this study, with one strain unable to grow at the lowest selecting temperature and the other exhibiting a supressed photosynthetic response after thermal selection. My findings highlight the complex relationship between symbiont identity, symbiont

thermal tolerance *ex hospite* and holobiont bleaching tolerance (Bhagooli and Hidaka, 2003; Gabay et al., 2018; Goulet et al., 2005). Here, the continuum of parasitism to mutualism of photosymbionts (Baker et al. 2018) is an important consideration in Symbiodiniaceae thermal selection experiments whereby the relative contribution of a symbiont to the host should be considered, for example, the amount of photosynthate produced and released, in addition to a strains ability to be taken up by the host and retained under heat stress.

It is important to consider the traits that are assessed during Symbiodiniaceae evolutionary experiments. Here I use growth rate as well as photosynthetic efficiencies as key traits. However, additional, or alternative traits may be required to better predict the fitness of corals inoculated with laboratory-evolved Symbiodiniaceae. For example, fast growth rate, although an important indicator of fitness, may not be advantageous to a symbiont, or its host, *in hospite.* Additionally, in terms of photophysiology, a recent study showed that corals containing *Durusdinium* (ITS2 type D1a) were able to retain a symbiosis at 33°C despite significant losses in photosynthetic function, while those harbouring *Cladocopium* (ITS2 type C3)also experienced photodamage but in contrast, they lost 99% of their symbionts (Silverstein et al. 2017).

It is also important that for temperature-selected Symbiodiniaceae in evolutionary experiments, I test their contribution to the host under heat stress, as well as their symbiotic performance at ambient temperatures – not just their ability to infect and be retained. For instance, following a bleaching event on the GBR in 2006, bleached *Acropora millepora* colonies harbouring *Cladocopium* were able to recover lipid levels to those of unbleached *Cldocopium* colonies, however *Durusdinium* colonies that had remained unbleached had 26% lower stored lipids compared to *Cladodopium* colonies with 28% smaller eggs and did not recover pre-bleaching lipid levels for nine months (Jones and Berkelmans 2011). In such cases, perhaps factors such as the extracellular production of ROS by the Symbiodiniaceae or lack of photosynthate release contributed the fitness of the holobiont. Studies investigating Symbiodiniaceae carbon fixation, release and incorporation into host tissue though radioactive ^{14}C labelling and stable isotopic ^{13}C and ^{15}N labelling (Baker et al., 2013, 2018; Biel et al., 2007; Cantin et al., 2009; Matthews et al., 2017; Pernice et al., 2015; Stat et al., 2008) together with newer technologies such as nanoscale secondary ion mass spectrometry (NanoSIMS) (e.g. Wangpraseurt et al., 2016) will be crucial for understanding the relative function of the symbiont within the host. This, along with measuring other physiological parameters relating to coral fitness response when harbouring laboratory-evolved Symbiodiniaceae as well as gene expression studies (e.g. DeSalvo et al., 2010; Pinzon et al., 2015; Traylor-Knowles et al., 2017), will thus be informative.

Finally, inoculation of aposymbiotic corals with a mix of experimentally evolved Symbiodiniaceae genera and species remains an important consideration. For example, it is possible that the host environment and/or nutritional needs of corals change throughout their life history such that different members of the Symbiodiniaceae confer different advantages depending on its hosts' life stage (Jones et al. 2008; Abrego et al. 2008; Quigley et al. 2016). Furthermore, absolute specificity is rare in corals (e.g. Quigley et al. 2014; Silverstein, Correa, and Baker 2012) and thus infecting corals with a cocktail of thermally selected strains could allow the host to retain those most beneficial under thermal stress through symbiont genotype shuffling (see Thornhill et al. 2006; Chen et al. 2005; Berkelmans and van Oppen 2006; Mieog et al. 2007), (See Berkelmans and van Oppen, 2006; Chen et al., 2005; Mieog et al., 2007; Thornhill et al., 2006) aiding in a flexible response to environmental change. Additionally, it is important that thermal tolerance of *ex-hospite* Symbiodiniaceae is not overlooked. Eighty five per cent of scleractinian coral species are broadcast spawners, of which 80% acquire symbionts horizontally, from free-living populations (Baird et al. 2009). It is therefore critical that the free-living Symbiodiniaceae pool remains thermally tolerant, as an exogenous source for coral larvae and recruits as well as bleached colonies (Boulotte et al. 2016) Furthermore, it is important to identify the refuges and associated other environmental conditions for symbionts during and after bleaching events . My results show that rapid adaptation is indeed possible in free-living Symbiodiniaceae populations undergoing directed selection and I show that this is possible across multiple genetically distinct strains of Symbiodiniaceae.

Conclusions and next-steps

After only one year of thermal selection and as few as 41 generations, I was already able to observe stable adaptive responses to elevated temperature in three of the five strains of Symbiodiniaceae included in this study. The observed increases in growth rates are comparable with evolutionary experiments in other microalgae, where selected populations have been exposed to elevated temperatures for up to 460 generations. A further year of thermal selection and I would expect to see the divergence in thermal tolerance of selected populations compared with ancestral ones to expand.

Important next-steps are to thoroughly investigate the infectivity of these strains under different temperature conditions, their stability *in hospite*, their effect on holobiont bleaching tolerance and health across different coral life stages, and to understand if and why thermal tolerance differs *ex-* and *in hospite*. Having knowledge of trait trade-offs and correlations such as heat tolerance and

carbon translocation, would be very useful to optimise a strategy for selection of symbionts. Such experiments will provide insight into which genera and species of the Symbiodiniaceae will be better sources for evolutionary experiments and the evolutionary rescue of corals *in situ* and which traits should be targeted in directed selection experiments that aim to develop symbionts with the ability to increase coral bleaching tolerance.

Chapter 5: Thermal and herbicide tolerances of chromerid algae and their ability to form a symbiosis with corals

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5.1 Summary

Reef-building corals form an obligate symbiosis with photosynthetic microalgae in the family Symbiodiniaceae that meet most of their energy requirements. This symbiosis is under threat from the unprecedented rate of ocean warming as well as the simultaneous pressure of local stressors such as poor water quality. Only 1 °C above mean summer sea surface temperatures (SSTs) on the Great Barrier Reef (GBR) can trigger the loss of Symbiodiniaceae from the host, and very low concentrations of the most common herbicide, diuron, can disrupt the photosynthetic activity of microalgae. In an era of rapid environmental change, investigation into the assisted evolution of the coral holobiont is underway in an effort to enhance the resilience of corals. Apicomplexan-like microalgae were discovered in 2008 and the phylum Chromerida (chromerids) was created. Chromerids have been isolated from corals and contain a functional photosynthetic plastid. Their discovery therefore opens a new avenue of research into the use of alternative/additional photosymbionts of corals. However, only two studies to-date have investigated the symbiotic nature of *Chromera velia* with corals and thus little is known about the coral-chromerid relationship. Furthermore, the response of chromerids to environmental stressors has not been examined. Here I tested the performance of four chromerid strains and the common dinoflagellate symbiont *Cladocopium goreaui* (formerly *Symbiodinium goreaui,* ITS type C1) in response to elevated temperature, diuron and their combined exposure. Three of the four chromerid strains exhibited high thermal tolerances and two strains showed exceptional herbicide tolerances, greater than observed for any photosynthetic microalgae, including *C. goreaui*. I also investigated the onset of symbiosis between the chromerids and larvae of two common GBR coral species under ambient and stress conditions. Levels of colonisation of coral larvae with the chromerid strains were low compared to colonisation with *C. goreaui*. I did not observe any overall negative or positive larval fitness effects of the inoculation with chromerid algae vs. *C. goreaui*. However, I cannot exclude the possibility that chromerid algae may have more important roles in later coral life stages and recommend this be the focus of future studies.

5.2 Introduction

Tropical reef-building corals provide a structural basis for one of the most productive and biodiverse ecosystem on Earth (Connell 1978) that generates essential ecological goods and services (Moberg and Folke 1999) in an otherwise oligotrophic environment. These foundation species form an obligate symbiosis with photosynthetic microalgae belonging to the family Symbiodiniaceae (LaJeunesse et al. 2018), which they rely upon for most of their energy requirements *via* the translocation of photosynthetic products from symbiont to host (Muscatine and Porter 1977; Falkowski et al. 1984; Muscatine 1990). Coral reefs are under serious threat from a range of anthropogenic pressures including ocean warming driven by the unprecedented release of anthropogenic carbon dioxide into the atmosphere (Hoegh-Guldberg, 1999; Hughes et al., 2017; Pandolfi et al., 2011), as well as local pressures such as a degradation of water quality through terrestrial run-off into coastal systems (Fabricius 2005).

Reef-building corals live near their upper thermal limits (Berkelmans and Willis 1999) and above this threshold the association between corals and their photosynthetic symbionts, Symbiodiniaceae*,* breaks down. Most evidence to-date points towards the excessive production of reactive oxygen species (ROS) from heat- and light-driven disruption to photosynthesis as the underlying cause of this breakdown (Lesser, 1997; McGinty, Pieczonka, & Mydlarz, 2012c), although more recent studies suggest changes in the nutrient exchange between both partners may also play a role (Wooldridge 2009b, a; Wiedenmann et al. 2013; Pogoreutz et al. 2017). The dissociation between the Symbiodiniaceae and coral and resulting paling of coral tissues is a process known as coral bleaching (Glynn 1984). Without their algae the coral host will eventually die, and thus coral bleaching is a phenomenon often leading to mass coral mortality (Glynn 1984; Berkelmans et al. 2004). On the Great Barrier Reef (GBR), four mass bleaching events were triggered by unusually high summer ocean temperatures in 1998, 2002, 2016, and 2017, with the proportion of reefs in the latter event experiencing four times more bleaching than previous events (Hughes et al., 2017).

Herbicide additions into the marine environment through terrestrial weed control and antifouling paints also pose a growing threat to marine life (Lewis et al. 2009). On the GBR, summer coincides with monsoonal rainfall (Lough 2007; Kroon et al. 2012; Lough et al. 2015) and thus many reefs are prone to the simultaneous effects of herbicide and thermal stress and salinity fluctuations. Increases in the intensity and frequency of rain and storm events associated with climate change lead to episodes of heightened exposure of coastal systems to terrestrial run-off (Noyes et al., 2009).

Diuron is one of the most commonly applied herbicides in the catchments of the GBR (Shaw et al. 2010) and is of particular concern due to its long persistence, high mobility and potency (Owen et al. 2003; van Dam et al. 2012; Mercurio et al. 2016). Indeed, diuron is detected year-round in some parts of the GBR (Kennedy et al. 2012). Diuron targets photosystem II in the chloroplast of photosynthetic organisms by binding to the D1 protein. It inhibits electron transport (Jones et al. 2003) and chronic exposure has been directly linked to coral bleaching (Cantin et al. 2007; Jones & Kerswell, 2003; Jones et al. 2003). Furthermore, diuron exacerbates the negative effect of other stressors, such as elevated temperature, on coral reef species (Negri et al. 2011; van Dam et al. 2012).

The rapid rate of environmental change represents a mounting challenge for the stability and function of the ancient symbiosis between the Symbiodiniaceae and coral host, particularly given that thermal thresholds for corals are already regularly exceeded (Hughes et al., 2017). Shuffling of genotypes among Symbiodiniaceae communities dominated by more thermally tolerant strains can increase coral bleaching tolerance by 1-1.5°C (Berkelmans and van Oppen 2006b) but such shifts are generally temporally unstable (Jones et al. 2008). Furthermore, some corals show high levels of symbiont fidelity (Baker 2003; Fabina et al. 2012) and therefore have limited scope for symbiont shuffling. *Ex hospite* directed selection for thermally resistant Symbiodiniaceae that can subsequently be introduced into aposymbiotic corals has therefore been explored as an intervention strategy to assist corals in their adaptation to ocean warming (**Chapters 2 and 4;** van Oppen et al., 2015).

In addition to the Symbiodiniaceae, there is a diversity of eukaryotes associated with corals that include the apicomplexan-related lineages (ARLs), many of which are largely uncharacterized (Clerissi et al. 2018), but can occur in high prevalence across coral groups (Kirk et al. 2013b, a; Kwong et al. 2018) Further, the discovery of apicomplexan-like algae with a functional photosynthetic plastid that live in close association with corals potentially opens new opportunities for assisted evolution to take advantage of alternative sources of phototrophic energy. Alternative photosymbionts include *Chromera velia,* first isolated from the coral *Plesiastrea versipora* in Sydney Harbour and formally described as an apicomplexan-like alga in the phylum Chromerida (Moore et al. 2008). Another chromerid strain initially also identified as *C. velia* was isolated from the tropical coral *Leptastrea purpurea* at One Tree Island, southern Great Barrier Reef (Moore et al. 2008). This strain shares metabolic features and photosynthetic ability with *C. velia* and is phylogenetically closely related, but differs substantially in morphology, cell ultrastructure and life-history and was

later formally described as *Vitrella brassicaformis* (Oborník et al. 2012). While most species in the Apicomplexa are parasitic protists and contain an unpigmented remnant chloroplast, the chromerids *C. velia* and *V. brassicaformis* contain a functional photosynthetic plastid, supporting the hypothesis that apicomplexans and dinoflagellates such as those belonging to the Symbiodiniaceae share a common ancestor (Gajadhar et al. 1991). Since their discovery, re-analyses of global microbial surveys found abundant apicomplexan-like eukaryotic sequences that were tightly associated with tropical corals, but in previous microbial surveys were mistaken as novel bacteria (Janouškovec et al. 2012). The association of chromerids and other apicomplexan-related lineages with healthy corals suggests a potentially mutualistic relationship between these algae and cnidarians, although further investigation into the frequency and extent of this association is needed.

Despite its apparent prevalence, no studies have investigated the environmental tolerance of the chromerids, and only two short-term laboratory studies have investigated the onset of symbiosis between chromerids and coral. In the first study, three new *C. velia* cultures, isolated from GBR corals were found to colonise two species of GBR coral, *Acropora digitifera* and *A. tenuis*; *C. velia* cells were harboured within the larval endoderm and ectoderm, up to three days after exposure of the larvae to the algae (Cumbo et al. 2013). These findings were interpreted as evidence for an endosymbiotic relationship between *C. velia* and corals. In contrast, other studies have indicated apicomplexan-related lineages are found on coral surfaces (Janouškovec et al. 2012) or exclusively in coral biogenous sediments and not in coral tissues (Mathur et al. 2018). The second study exposed larvae of the tropical coral, *A. digitifera,* to the *C. velia* strain that was isolated from *Plesiastrea versipora* in Sydney Harbour (Moore et al. 2008), and compared the transcriptomic response of the inoculated larvae with that of non-inoculated control larvae for up to two days after their introduction (Mohamed et al. 2018). Genes involved in a typical host response to harmful parasites were upregulated when corals were inoculated with *C. velia* that led the authors to conclude a non-mutualistic relationship exists between coral and *C. velia*. However, the uptake of *C. velia* by the coral host was not quantitatively measured, and the environmental differences at the collection locations of the *C. velia* and the coral could have contributed to the results of this study. Finally, the existence of host-symbiont specificity is well documented for the coral-Symbiodiniaceae symbioses, and similar specificity may exist for strains of the chromerids (Rodriguez-Lanetty et al. 2004; LaJeunesse et al. 2010; Smith et al. 2017). The absence of data on the physiological response of the chromerids to adverse environmental conditions along with the limited number of experiments investigating the onset of symbiosis between chromerid algae and corals means that it remains uncertain whether the coral-chromerid association can function as a mutualism.

From an assisted evolution view-point, the ability of *C. velia* (and *V. brassicaformis*) to photosynthesise, the prevalence and association of apciocomplexan-like sequences with corals and the successful short-term uptake of *C. velia* by two coral species, warrants further investigation into the apicomplexans as coral symbionts. Here I investigate the plastic response of three cultured strains of GBR *C. velia* and one strain of *V. brassicaformis,* to temperature and diuron and both stressors simultaneously, and compare this to the tolerance of a culture of the globally distributed *Cladocopium goreaui* belonging to the family Symbiodiniaceae (formerly known as *Symbiodinium goreaui,* with an ITS2 designation of type C1) (Trench and Blank 1987; LaJeunesse 2005). This alga is a host-generalist, commonly found in association with corals on the GBR (LaJeunesse et al. 2003, 2004). I also investigate the stability of the association of each microalgal strain with larvae of two GBR coral species, as well as larval mortality, under heat and herbicide stress to assess whether any differences in heat and herbicide tolerances in culture transfer to the coral host upon their inoculation. My investigation explored the use of alternate symbionts, the Chromerida, to enhance the environmental stress tolerance of corals in a rapidly changing ocean.

5.3 Methods and Materials

Experimental microalgal strains

Cladocopium goreaui, strain SCF055-01.10, was isolated from the coral *A. tenuis,* Nelly Bay, Magnetic Island, Australia (**Table 5.1**) (19°1006"S, 146°50060"E) in 2010 and a culture was maintained at 27°C and 65 ± 10 µmol photons m^{-2} s^{-1} (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle. Note that this strain is the same used in **Chapter 2**, and was derived from a single cell isolated from a heterogeneous culture of cells extracted from the coral host, investigated in two prior studies (Howells et al., 2012; Levin et al., 2016), however, this strain was then referred to as *Symbiodinium goreaui* type C1 (**Table 5.1**).

An unidentified alga (strain SCF055-02) was obtained from the original heterogeneous *C. goreaui* culture that grew unexpectedly in a replicate culture from the coral extract (**Table 5.1**). This occurred during a mutagenesis experiment where 256 replicate cultures were exposed to the chemical mutagen ethyl methane sulphonate (EMS), based upon methods from (Chaturvedi and Fujita 2006) and subsequently exposed to 100 µg L^{-1} of diuron. This experiment was designed to select for mutation(s) that conferred diuron tolerance in *C. goreaui.* The replicate cultures were checked monthly for any indication of growth, using light microscopy and pulse-amplitude modulated fluorometry. After approximately one year, one of 256 replicate culture vessels showed

signs of live cells. After subsequent, continuous re-inoculation into freshly prepared, sterile culture media, Daigo's IMK for Marine Microalgae (Nihon Pharmaceutical Co., Ltd) containing diuron, this replicate culture remained alive and showed growth. This diuron-resistant strain was given the identification SCF055-02. This replicate was subsequently cultured with 30 μL^{-1} of diuron for 2.5 years, with monthly sub-culturing into fresh media. To investigate its genetic identity, I extracted DNA using the Wayne's method (Wilson et al. 2002) and amplified the 18S rDNA small subunit region using the universal eukaryotic Forward (ss5 – 5'- GGTTGATCCTGCCAGTAGTCATATGCCTTG - 3') and Reverse (ss3 – 5'- GATCCTTCCGCAGGTTCACCTACGGAAACC - 3') primers (Rowan and Powers 1992; Cumbo et al. 2013) to give a PCR product of ˜1800 bp in size. PCR amplification was performed using 2 ng of genomic DNA as the template and with 12.5 µL of mastermix (Qiagen) and 5 µL of each primer. Conditions for amplification were the same as in Cumbo et al. (2013). PCR products were Sanger sequenced in both directions at the Australian Genomics Research Facility (AGRF). Forward and reverse sequences were assembled in Sequencher (Version 5.4.5) and BLASTn (database: nucleotide collection nr/nt) searches of the assembled sequences were conducted at blast.ncbi.nlm.nih.gov.

The top 10 blast matches were all to the chromerids *C. velia* and *V. brassicaformis* (**Appendix 5.3**). Two GBR cultures of chromerids, Mdig2 and Mdig3, assumed to be *Chromera velia* (from Cumbo et al., 2013) were obtained (**Table 5.1**), and 18S sequences downloaded from Genbank (Accession numbers JN986789.1 and JN986790.1, respectively). One strain of *V. brassicaformis* was sourced (originally from Moore et al., (2008) and later described by Oborník et al. (2012)) and also 18S sequenced, as before. To identify my unknown chromerid as either *C. velia* or *V. brassicaformis* I aligned the four 18S chromerid sequences in Sequencher (version 5.2.4) and found only one base pair different between the unknown chromerid and *V. brassicaformis* and five and four base pairs different with Mdig2 and Mdig3, respectively (renamed *C. velia1* and *C. velia2* henceforth). Phylogenetic analyses were carried out in Mega (version 7.0.18) and were inconclusive due to the similarity of each of the four chromerid sequences (**Appendix 5.1**).

Table 5.1. Species, host species and site of origin of each microalgal strain used in this study

Strain identification	Species	Host species	Geographic origin	Original study
SCF055-01.10	*Cladocopium goreaui* (formerly *Symbiodinium* goreaui: ITS type C1)	*Acropora tenuis*	Magnetic Island (Central GBR)	**Chapter 2**
Mdig2 (Named "*C. velia1"* in this study)	*Chromera velia*	*Montipora digitata*	Magnetic Island (Central GBR)	Cumbo et al. 2013
Mdig3 (Named "*C. velia2"* in this study)	*Chromera velia*	*Montipora digitata*	Magnetic Island (Central GBR)	Cumbo et al. 2013
CvLp_vc08/1, CCMP3155 or CMS22 (named *Vitrella brassicaformis* in this study)	*Vitrella brassicaformis* (formally known as *C. velia)*	*Leptastrea purpurea*	One Tree Island (Southern GBR)	Moore et al. 2008; Oborník et al., 2012 and more
SCF055-02 (named "unknown chromerid" in this study)	Unknown (tentatively *V. brassicaformis*)	*Acropora tenuis*	Magnetic Island (Central GBR)	This study

Microscopic observations revealed the most similar morphologies of SCF055-02 with *V. brassicaformis* compared to the two *C. velia* strains (**Appendix 5.2**). Further genetic and morphological analyses are required to confirm this. For the purpose of this study I name SCF055-02 as the "unknown chromerid". Thus, from here forth the four chromerid strains are described as *C. velia1* and *C. velia2* (strains Mdig2 and Mdig3, respectively), *V. brassicaformis* and the unknown chromerid strain (**Table 5.1**).

***In vitro* temperature and diuron sensitivity of *Cladocopium goreaui* and the chromerids**

Firstly, to test the sensitivity of *C. goreaui* and chromerid strains to temperature, diuron and their combined effects, I carried out dose-response toxicity assays using 11 diuron concentrations (0, 0.3, 1, 3, 10, 30, 100, 300, 1000, 3000 and 5000 µg L^{-1}- values based on preliminary diuron-tolerance experiments) and four temperatures (27°C control, 30°C, 31°C and 32°C, **Appendix 5.4**). This temperature range was chosen based on previous findings that the strain of *C. goreaui* used in this

study cannot survive at 31°C or beyond for a prolonged period of time (**Chapter 2).** The highest temperature treatment of 32°C was chosen because the thermal sensitivities of the chromerids were unknown. Experiments took place using four temperature-controlled environmental chambers (Steridium, er-rh-500) with 65 ± 10 µmol photons.$m^{-2}.s^{-1}$ (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle and temperature measurements were recorded every 10 min, using a data logger (HOBO Pendant, **Appendix 5.4**).

Cells were pre-acclimated to the four temperatures, for either 10 or 20 days prior to dosing with diuron, to test whether the duration of temperature pre-exposure had an effect on thermal sensitivity, or an interactive effect with diuron. Ten and 20-day pre-acclimation durations were based on previous experiments with the *C. goreaui* strain used in this study that demonstrated no negative photosynthetic effects after 13 days at 32°C (Levin et al. 2016) but negative physiological effects of 31°C after 17 days (**Chapter 2)**.

A stock solution of diuron was prepared by dissolving analytical grade diuron (Sigma Aldrich, > 95% pure) in 100 % ethanol. The 11 concentrations were prepared at double concentration in IMK medium, using the stock diuron solution as well as an ethanol solvent control solution (carrier only, 0.003 % (v/v) final concentration), representing the highest ethanol concentration in the 5000 µg L^{-1} diuron treatment. Fifty µL of each of the 11 diuron concentrations and solvent control were transferred into triplicate black, clear-bottom 96-well culture plates (Costar, Corning®, Sigma-Aldrich) in a randomised well design. Temperature pre-acclimated cultures were pelleted (295 g/2000 rpm, 5 min), media removed, and cells resuspended in fresh IMK media at a cell density twice that required for the diuron dosing (3 200 000 cells mL^{-1}). Fifty µL of each culture were subsequently added to each well to give a final concentration of 1 600 000 cells mL^{-1} and with a final volume of 100 µL. This resulted in nine replicate wells for each microalgal culture, spread across triplicate plates. Plates were placed in their respective temperature conditions under 60 µmol photons $m^{-2}s^{-1}$ for 48 h before chlorophyll a fluorescence measurements were taken. The effective quantum yield in an illuminated plant [$\Delta F/F_m' = (F_m' - F')/ F_m'$] provides an estimate of the efficiency of photochemical energy conversion within photosystem II under a given light intensity (Genty et al. 1989). The reversible binding of photosystem II herbicides to the D1 protein in photosystem II results in an acute and temporary reduction in $\Delta F/F_m'$ (Jones and Kerswell, 2003). The maximum quantum yield [$F_v/F_m = (F_m - F_0) / F_m$] is equivalent to the proportion of light used for photosynthesis by chlorophyll when all reaction centres are open (Genty et al. 1989) and reductions in Fv/Fm indicate inactivation and/or photo-oxidative damage to photosystem II (chronic photoinhibition) (Schreiber 2004). Finally, I

measured the maximum excitation pressure over photosystem II, Q_m = 1- [($\Delta F/F_m'$)/ F_v/F_m)], where values close to zero indicate that even during periods of the maximum irradiance most reaction centres remain open, suggesting that photosynthetic rates are light-limited. However, values close to 1.0 indicate that under maximum irradiance most of the photosystem reaction centres are closed, suggesting photoinhibition (Iglesias-Prieto et al. 2004).

Maximum quantum yield measurements were taken one hour before the end of the dark cycle, and effective quantum yield measurements were attained by subsequently pre-acclimating plates to six minutes of an actinic light of two (PAR=20 units) before applying a saturation pulse, methods based on (Schreiber et al. 2007). Measurements for effective quantum yields were carried out twice in close succession and a mean value for each replicate obtained (nb. a preliminary study showed that there were no significant differences between mean effective quantum yield values of cells exposed to six minutes of actinic light once and cells exposed to six minutes of actinic light six times, with saturation pulses in between).

The results from 0 µg L^{-1} of diuron in these experiments were used to investigate the effects of temperature alone on the photochemistry of these microalgae. Therefore, results here reflect a total of 12 and 22 days temperature exposure. The maximum quantum yields (F_v/F_m) and pressure over photosystem II, (Q_m) are reported only for the effect of temperature on the different microalgal strains.

Coral larval inoculation with *Cladocopium goreaui* and chromerids

To determine the relative colonisation abilities of *C. goreaui* and the four chromerid strains, as well as their effect on the coral host, I introduced the microalgae to aposymbiotic larvae of two coral species, *Acropora tenuis* and *Acropora millepora.* Colonies of *A. tenuis* were collected from Falcon Island (18°46' E 146°32' S) in November 2017, while colonies of *Acropora millepora* were collected from Backnumbers Reef (18°29'264"E 147°09'174"S) in December 2017. Both species were kept in the National Sea Simulator at the Australian Institute of Marine Science (Townsville, Australia) for three days before the full moon. Following spawning, gametes were collected from six colonies of each species and gametes from the same species were mixed, using approximately equal sperm concentrations (~10^6 mL^{-1}) for fertilisation. Resulting larvae were kept in aerated, flow-through 0.4 µm filtered seawater, for 12 and 37 days before the experiment for *A. tenuis* and *A. millepora*, respectively.

Cladocopium goreaui and the chromerid cultures were pre-acclimated to three temperature treatments of 27°C, 30°C and 31°C (**Appendix 5.4**) for two weeks before being introduced to the coral larvae. Larvae were pre-acclimated for three days to each temperature treatment (**Appendix 5.5**). From previous observations, the uptake of *C. goreaui* at 31°C for *A. tenuis* and *A. millepora* has been minimal and thus I chose 31°C as the maximum temperature treatment for this *in hospite* experiment. Ten larvae were added to each well of triplicate, six-well plates (Corning, Sigma-Aldrich) containing 9 mL of *C. goreaui* or chromerid algae in filtered seawater, at a concentration of 15 000 cells mL^{-1} and containing either 30 µg L^{-1} of diuron or 0 µg L^{-1} diuron. Plates were placed at the three temperatures under 60 µmol photons $m^{-2}s^{-1}$. This experimental setup resulted in 180 larvae *per* diuron treatment *per* temperature, for each coral species. Two days later, a near-full water change was conducted in each well. Subsequently, every two days, the salinity in one randomly chosen well of each plate was measured and water changes were conducted for all wells if the salinity had increased. Nb. While plates were sealed to prevent evaporation, condensation was minimal and did not become a problem.

Fourteen days after their exposure to *C. goreaui* and the chromerids, one larva *per* well was removed, placed on a microscope slide and viewed under a fluorescent microscope (Zeiss Axioscop2 molt plus) using the filter (Zeiss F. set09 (FITC), ex 495nm em 517 nm) allowing the algal cells to be clearly differentiated from the host cells by observing the red autofluorescence of chlorophyll within the algae. For each temperature, diuron treatment and larval species, the total number of algal cells hosted by the larvae was counted (n=18 larvae across three triplicate plates), with larvae containing one or more algal cells being recorded as 'colonised'. The proportion of colonised larvae was also calculated (n=3: the proportion of colonised larvae that were removed from each plate, across triplicate plates). Finally, the number of larvae remaining in each well at each time point was recorded to assess mortality (n=18 wells across triplicate plates). Any larvae that had metamorphosed were removed from the wells, but still counted as alive. After 14 days, remaining larvae were fixed in 2.5% glutaraldehyde and stored at 4°C for visualisation on a confocal microscope.

Confocal microscopy

Slides were prepared with larvae, using a mounting solution of 90 % glycerol, 10 % phosphate buffer solution (PBS) and were visualised using a laser scanning confocal microscope (Zeiss 710) using an EC Plan-Neoflaur 20x/0.50 M27 objective with a pinhole (depth resolution) of 28 µm, emission

wavelengths of 435-726 nm with the following excitation wavelengths and intensity: 405 nm: 60%, 561 nm: 45 % and 488 nm: 55%. The master gain was set at 476 units (**Figure 5.6**).

Statistical analyses

In vitro

To test the effects of diuron concentration and the interaction of diuron and temperature on the photochemistry of *C. goreaui* and the chromerids, effective quantum yield data were fitted with a variable slope log(dose) vs. response curve and the EC50 values (the concentration of diuron that gives half-maximal response) were interpolated from the non-linear fits. Confidence intervals (95%) were calculated for each mean EC50 value and compared for each microalgal strain. I considered the overlap of 95% confidence intervals between means as the means not being significantly different and *vice versa.* Analyses were carried out using GraphPad Prism (v. 7.03). The effects of temperature and strain on photophysiological traits were tested using linear mixed effects models with plate as a random factor. Analyses were performed in R (v. 3.4.1; R Core Team, 2017) using the packages, nlme (v. 3.1-131) and multcomp (v. 1.4-8).

In hospite

To test the effects of microalgal strain, diuron and their interaction on the uptake of microalgae and mortality in two species of coral larvae, I carried out general linearised models with Poisson (uptake) and binomial (mortality) distributions. Where there were no significant interactions between strain and diuron, the interaction term was removed and run as an additive model; model choice was confirmed using the Akaike Information Criterion (AIC). Post-hoc analyses were carried out using Tukey's tests ($p > 0.05$) and when a significant strain by diuron interaction was evident, a planned comparison matrix was carried out. This involved comparing uptake/mortality between all strains on larvae inoculated with each strain. For mortality data, mean proportion values were calculated from differing replicate numbers, therefore replicate weights were specified in the model. Separate analyses were carried out for both traits (larval uptake and larval mortality) at the three temperature conditions, 27, 30 and 31 °C. Analyses were carried out in R (v. 3.4.1; R Core Team, 2017) using the following packages; lme4 (v. 1.1-17), car (v. 3.0-0), MASS (7.3-47), multcomp (v. 1.4-8) and MuMIn (v. 1.42.1).

5.4 Results

In vitro experiments

A series of *in vitro* experiments demonstrated that temperature alone, diuron alone and the simultaneous exposure of temperature and diuron affected the photochemistry of all microalgae tested and that there were striking differences in response between strains.

Effect of temperature on photochemistry

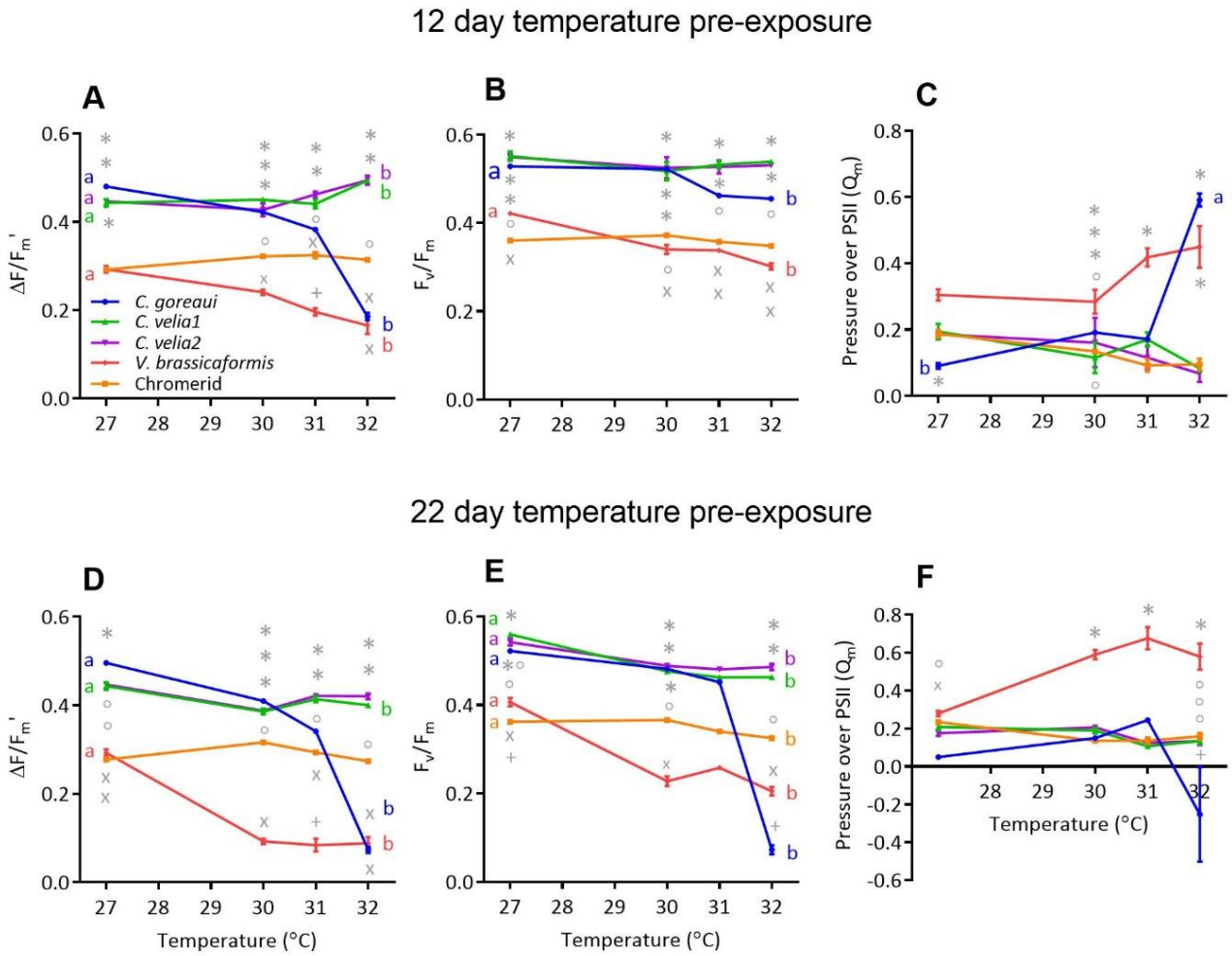

Figure 5.1. The effect of 12 days (A-C) and 22 days (D-F) of exposure to different temperatures on the (a, d) effective ($\Delta F/F_m'$) and (b, e) maximum (F_v/F_m) quantum yields and (c, f) pressure over photosystem II of *Cladocopium goreaui* and four chromerid strains. Different symbols represent statistically significant differences in photochemistry between strains within the same temperature treatment. Different lowercase letters represent statistically significant differences between photophysiological traits at 27 °C and 32 °C Data points represent means (n=9) ±SE

Strain, temperature and their interaction had a significant effect on microalgal effective ($\Delta F/F_m'$) and maximum (F_v/F_m) PSII quantum yields and pressure over photosystem II (Q_m) after both 12 and 22 days of temperature exposure (**Appendix 5.6**). Post-hoc comparisons indicated that there were

differences in both $\Delta F/F_m'$ and F_v/F_m at the control temperature of 27 °C between the microalgal strains (**Figure 5.1**); *Cladocopium goreaui*, *C. velia1*, *C. velia2* all had significantly greater mean yields compared to *V. brassicaformis* and the unknown chromerid, that were up to 60 % greater in $\Delta F/F_m'$ and 65% in F_v/F_m (max $p < 0.01$). After both durations of temperature exposure, *C. goreaui* experienced the largest decline in both F_v/F_m and $\Delta F/F_m'$ at the highest temperature treatment, falling by as much as 85-86% after 22 days and 14- 57% after 12 days at 32 °C, respectively (**Figure 5.1b**, max $p < 0.01$). This was accompanied by an 85% increase in mean Q_m after 12 days temperature exposure at 32 °C (**Figure 5.1c**, $p < 0.01$), results that were no longer apparent after 22 days due to $\Delta F/F_m'$ and F_v/F_m becoming equally low (**Figure 5.1F**, $p = 0.06$).

ΔF/Fm' and Fv/Fm also decreased for *V. brassicaformis* with increasing temperature (**Figure 5.1a, b**, **d, e**, $p < 0.01$) and this species exhibited the highest Q_m values at 31 °C after 12 days (**Figure 5.1c**, max. $p = 0.046$) and the highest at 30, 31 and 32 °C after 22 days (**Figure 5.1F**, max. $p < 0.01$)). *C. velia1*, *C. velia2* and the unknown chromerid on the other hand were able to maintain stable ΔF/Fm' and Fv/Fm and Q_m value across the temperature conditions after 12 days of exposure (**Figure 5.1 a, b, c**). After 22 days of exposure at 32 °C, there was a small but statistically significant decrease by 9.6% in $\Delta F/F_m'$ for *C. velia1* compared to 27 °C (**Figure 5.1d**, $p < 0.01$) while all three strains exhibited statistically significant decreases, by 17%, at the most, in F_v/F_m at 32 °C compared to 27 °C (max. $p < 0.01$).

Effect of diuron on photochemistry

There were significant differences in the effect of diuron on $\Delta F/F_m'$ between different microalgal strains (**Table 5.2**). *Cladocopium goreaui* was the most sensitive species, exhibiting the lowest EC50, approximately 4-fold and 3-fold lower than the EC50 values for *C. velia1* and *C. velia2* respectively (**Figure 5.2a**, **Table 5.2**). *Vitrella brassicaformis* and the unknown chromerid were highly insensitive to diuron (**Figure 5.2a**), requiring over a 130-fold higher diuron concentration for 50% ΔF/Fm' inhibition in comparison to *C. goreaui*. (**Table 5.2**).

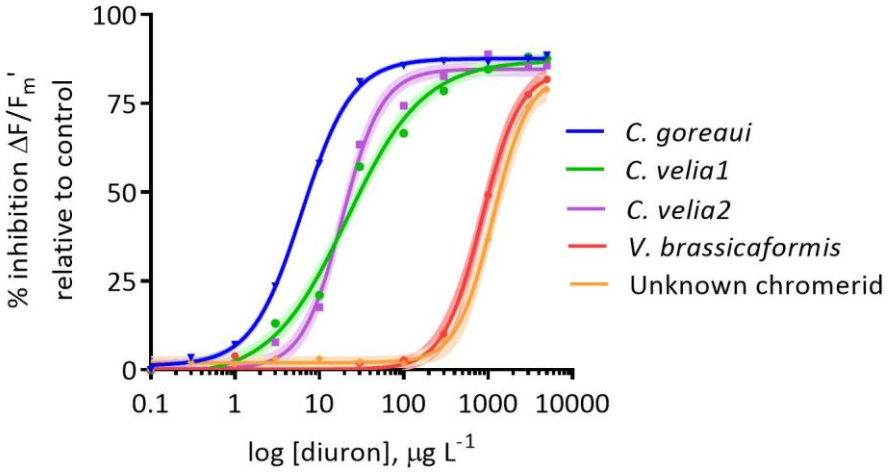

Figure 5.2. Dose-response of the percentage inhibition of the effective ($\Delta F/F_m'$) quantum yields of *Cladocopium goreaui* and four chromerid strains exposed for 48 h to diuron at 27°C. Inhibition is relative to the quantum yield values at 0 µg $L^{-1}$of diuron. Curves are fitted with non-linear regressions. Data points represent means (n=9). The narrow-shaded areas represent confidence interval (95%) bands.

Table 5.2. EC50 values and 95% confidence intervals (CI) for effective ($\Delta F/F_m'$) quantum yields after 48 h of exposure to diuron at 27°C

	ΔF/Fm'	
Microalgal strain	EC50 (µg L^{-1})	CI range
Cladocopium goreaui	7.4	7.2-7.7
Unknown chromerid	1365	1226-1551
Chromera velia1	30	27-35
Chromera velia2	22	21-25
Vitrella brassicaformis	1024	930-1134

Effect of combined diuron and temperature on photochemistry after 12 days of temperature exposure

Temperature significantly affected the diuron sensitivity of ΔF/Fm' across all of the microalgal strains after 12 days of exposure to 32 °C (**Figure 5.3**, **Tables 5.3, 5.4**). *Cladocopium goreaui* experienced more than 50 % inhibition in $\Delta F/F_m'$ at 32 °C before any diuron additions (**Figure 5.3a**) and thus an EC50 value was not calculated (**Table 5.3**). The $\Delta F/F_m'$ of *V. brassicaformis* was also strongly inhibited by temperature in the absence of diuron and this inhibition was greater after 22 d of elevated temperature in comparison to the 10 d exposure (Fig. 3G-H, **Table 5.3**). The negative effects of diuron on $\Delta F/F_m'$ increased at 32°C in *C. velia1* and *C. velia2* with EC50s reached at 18 – 43% lower diuron concentrations, and these negative impacts were also greater for the longer 22 d exposures

(Fig. 3C-F, **Table 5.3**). Interestingly the unknown chromerid became significantly less sensitive to diuron at elevated temperature as shown by the increase in EC50s at 32°C by 25 and 61% after 10 and 22 days, respectively (**Figure a,b**, **Table 5.3**). At low diuron concentrations the unknown chromerid, *C. velia1* and *C. velia2* all exhibited higher photosynthetic efficiencies (were less inhibited) at 32 °C than at 27°C (Fig. 3 A,C,D,E).

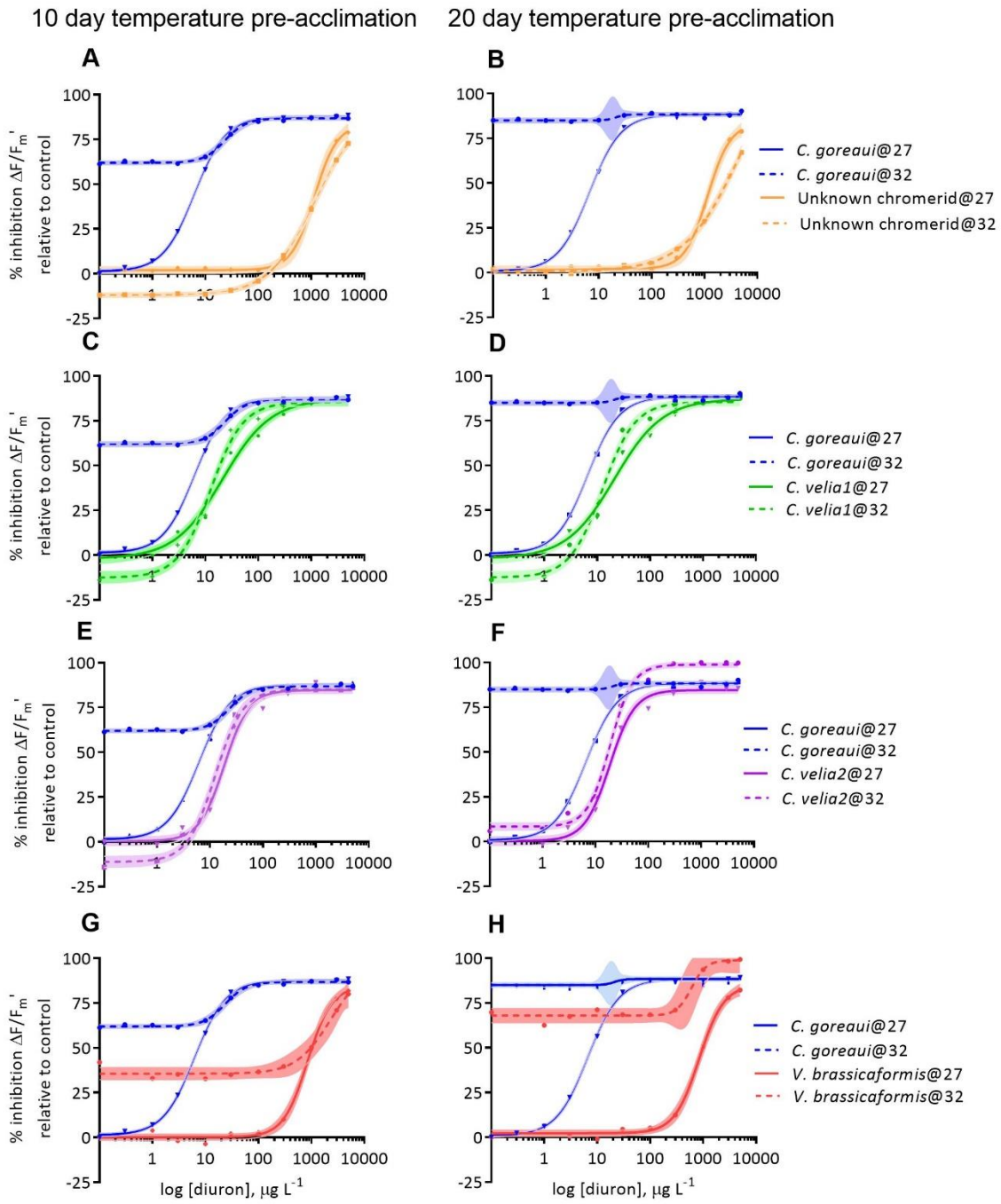

Figure 5.3. Dose-responses to diuron of the percentage inhibition of effective ($\Delta F/F_m'$) quantum yields of *Cladocopium goreaui* and four strains of chromerids at 27°C and 32°C, after 10 and 20 days of exposure to each temperature before being exposed to diuron for 48 h and thus spending a total of 12 and 22 days exposed to the different temperature conditions. Inhibition is relative to the quantum yield values at 0 ug L^{-1} of diuron and 27°C for each strain. Curves are fitted with non-linear regressions. Shaded areas represent confidence interval (95%) bands.

Table 5.3. EC50 values and 95% confidence intervals (CI) for effective ($\Delta F/F_m'$) quantum yields after 10 and 20 days of pre-acclimation to 27°C or 32°C and a further 48 hours post diuron-dosing and % changes in EC50 values with temperature. *lower CI was not able to be calculated

		27°C		32°C		
Temp. pre-acclimation (days)	Microalgal strain	EC50 ($\mu g\ L^{-1}$)	CI range	EC50 ($\mu g\ L^{-1}$)	Upper CI	% change
10	*Cladocopium goreaui*	7.4	7.2-7.7	already >50% inhibited		NA
20		7.9	7.6-8.1	already >50% inhibited		NA
10	Unknown chromerid	1365	1702-1551	1702	1578-1832	+25
20		1365	1226-1551	2592	2292-2892	+61
10	*Chromera velia1*	30	19-35	19	17-21	-37
20		30	27-35	17	17-21	-43
10	*Chromera velia2*	22	18-25	18	16-20	-18
20		23	21-25	17	16-19	-26
10	*Vitrella brassicaformis*	1024	971-1134	971	561-1505	-5
20		990	899-1097	already >50% inhibited		NA

***In hospite* experiments**

A series of *in hospite* experiments involving the introduction of the five microalgal strains to coral larvae of two species, *Acropora tenuis* and *Acropora millepora,* revealed differences in their uptake capability and mortality under diuron and with no diuron, across three temperature treatments.

Acropora tenuis

Larval uptake: At 27 °C and 30 °C the mean larval uptake of microalgae by *Acropora tenuis* was significantly affected by microalgal strain, diuron and their interaction. No other factor except for strain identity affected larval uptake at 31°C, although the means were low and differences were minimal (**Appendix 5.7**).

Post-hoc analyses revealed that at 27 °C the uptake of *C. goreaui* by larvae was more than 115 times greater than the next most successful chromerids, *C. velia1* and *C. velia2* (**Figure 5.4a**, $p < 0.01$). Diuron significantly reduced the uptake of *C. goreaui, C. velia1* and Mdig 3 at 27 °C (**Figure 5.4b**, $p < 0.01$), a trend that continued at 30 °C for *C. velia1* and *C. velia2*. In contrast *C. goreaui* uptake was 15 times greater in the presence of diuron 30 °C (**Figure 5.3b**, $p < 0.01$).

Larval mortality: At 27 °C, both strain and diuron had statistically significant effects on larval mortality (**Figure 5.4d**, **Appendix 5.7**), whereby the three strains that had the highest uptake, *C.*

goreaui, *C. velia1* and Mdig 3, also exhibited the greatest larval mortality, compared with the remaining strains (**Figure 5.4d**, $p < 0.03$). At 30 °C, only the presence of diuron significantly affected larval mortality **(Appendix 5.7**), with an overall trend of increased mortality with diuron (**Figure 5.4e**), again reflecting a similar trend to larval uptake. At the highest temperature treatment of 31 °C, larval mortality was significantly affected by algal strain, diuron and their interaction (**Figure 5.4f**, **Appendix 5.7**) and did not follow larval uptake trends. Specifically, under 0 µg/L of diuron, larvae exposed to the unknown chromerid had the highest mean mortality, that was 10% greater than the next highest, *C. goreaui* (**Figure 5.4f**). Under 30 µg L^{-1} of diuron, larvae with *C. goreaui* experienced 20% mortality, which was significantly higher than larvae exposed to and colonised with the remaining microalgal strains (**Figure 5.4f**, $p < 0.01$). Furthermore, larvae harbouring *C. goreaui* were the only treatment at 31 °C to be significantly affected by diuron (**Appendix 5.6**), evidenced by an increase in mortality by 8% in the presence of diuron (**Figure 5.4f**, $p < 0.01$).

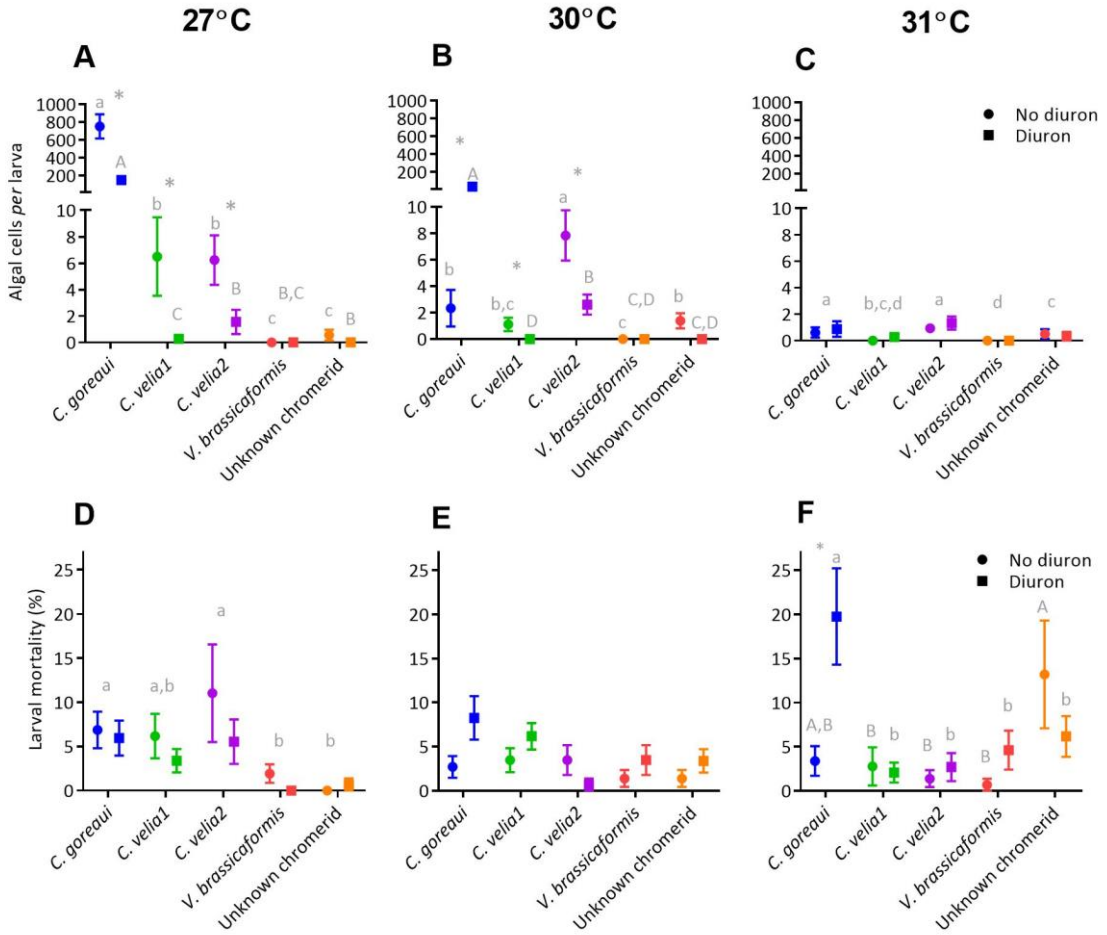

Figure 5.4. The effect of microalgal strain and diuron (30 µg L^{-1} diuron) on the uptake of microalgae and mortality of *Acropora tenuis* larvae at three temperature conditions at day 14 of the experiment. Different lower-case letters represent statistically significant differences between strains in the absence of diuron, while upper-case letters represent statistically significant differences between strains under 30 µg L^{-1} of diuron. * symbol represents a significant effect of diuron within a strain (Tukeys tests, $p < 0.05$). Data points represent means ± standard error.

Acropora millepora

Algal uptake by larvae: At all temperature treatments, the mean uptake of microalgae by *Acropora millepora* larvae was significantly affected by microalgal strain, diuron and their interaction (**Appendix 5.8**). Post-hoc analyses revealed that such effects were most likely driven by *C. goreaui* where mean uptake of *C. goreaui* at 27 °C and 30 °C was significantly greater than the mean uptake of the chromerids, under both diuron and no diuron conditions (**Figure 5.5a,b**, max. $p < 0.01$); chromerids all showed minimal colonisation capacity, with larvae in these treatments harbouring less than five symbionts *per* larva. Diuron significantly reduced the uptake of *C. goreaui* at all three temperature treatments (**Figure 5.5a**, b, c, max. $p = 0.005$), the greatest being a five times reduction in symbionts *per* larva at 27 °C (**Figure 5.5a**, $p < 0.01$).

Larval mortality: At 27 °C, both strain and diuron had statistically significant effects on larval mortality (**Appendix 5.8**), with a general trend of increased mortality under diuron conditions (**Figure 5.5d**). Post-hoc analyses indicated that larvae in the presence of *C. goreaui* and *C. velia2* had the highest mean mortalities (**Figure 5.5d**). At 30 °C there was a significant effect of diuron and the interaction of strain and diuron on larval mortality (**Appendix 5.8**). Although post-hoc analyses were unable to identify specific differences in pairwise comparisons, trends show an overall increase in mortality with diuron, especially for *C. goreaui* and *C. velia2*, where mean mortality increased by over 7% (**Figure 5.5e**). At 31 °C there was a significant effect on the interaction of strain and diuron on larval mortality (**Appendix 5.8**). Although post-hoc analyses were unable to disentangle these differences, trends indicate that mean mortalities surprisingly decreased with diuron for those larvae exposed to *C. goreaui,* and the unknown chromerid, while larvae with *V. brassicaformis* had increased mortality in the presence of diuron (**Figure 5.5f**).

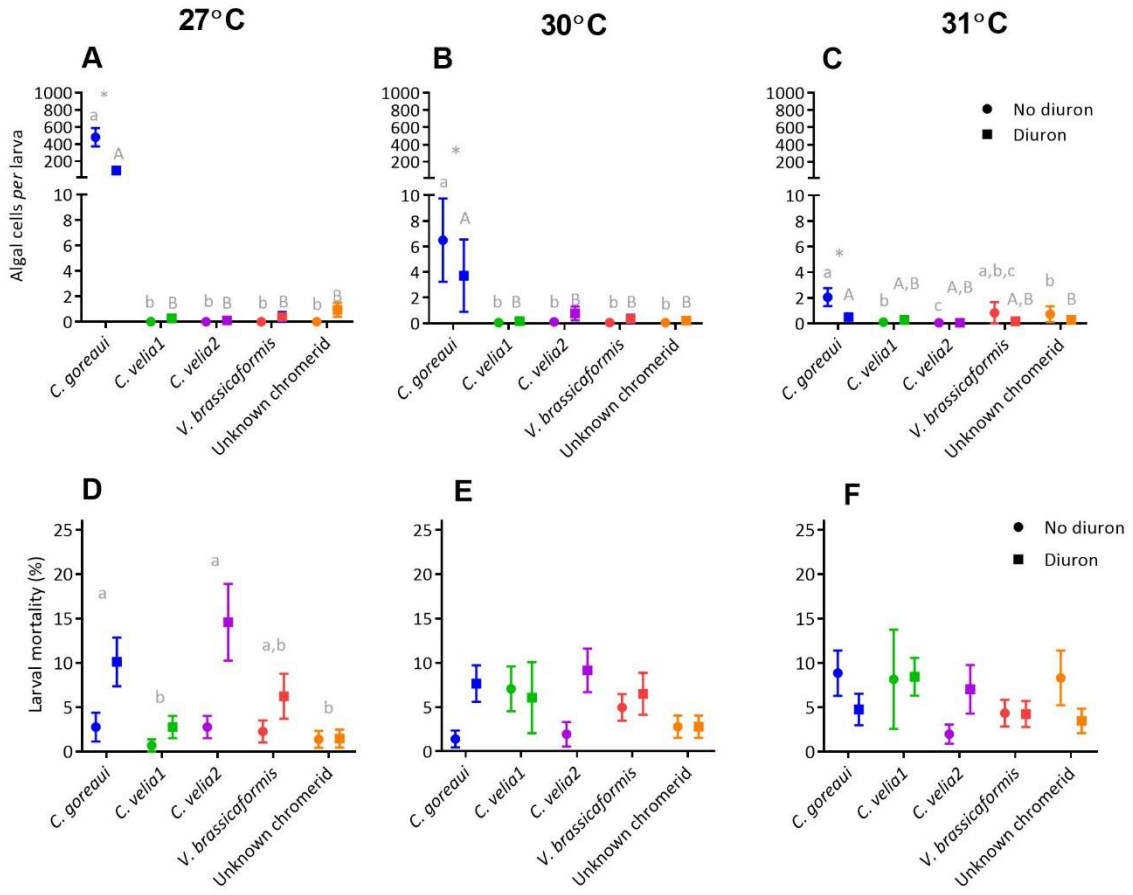

Figure 5.5. The effect of microalgal strain and diuron (30 µg L^{-1} diuron) on the uptake of microalgae and mortality of *Acropora millepora* larvae at three temperature conditions after 14 days. Different lower-case letters represent statistically significant differences between strains in the absence of diuron, while upper case letters represent statistically significant differences between strains under 30 µg L^{-1} of diuron. * symbol represents a significant effect of diuron within a strain (Tukeys tests, $p < 0.05$). Data points represent means ± standard error.

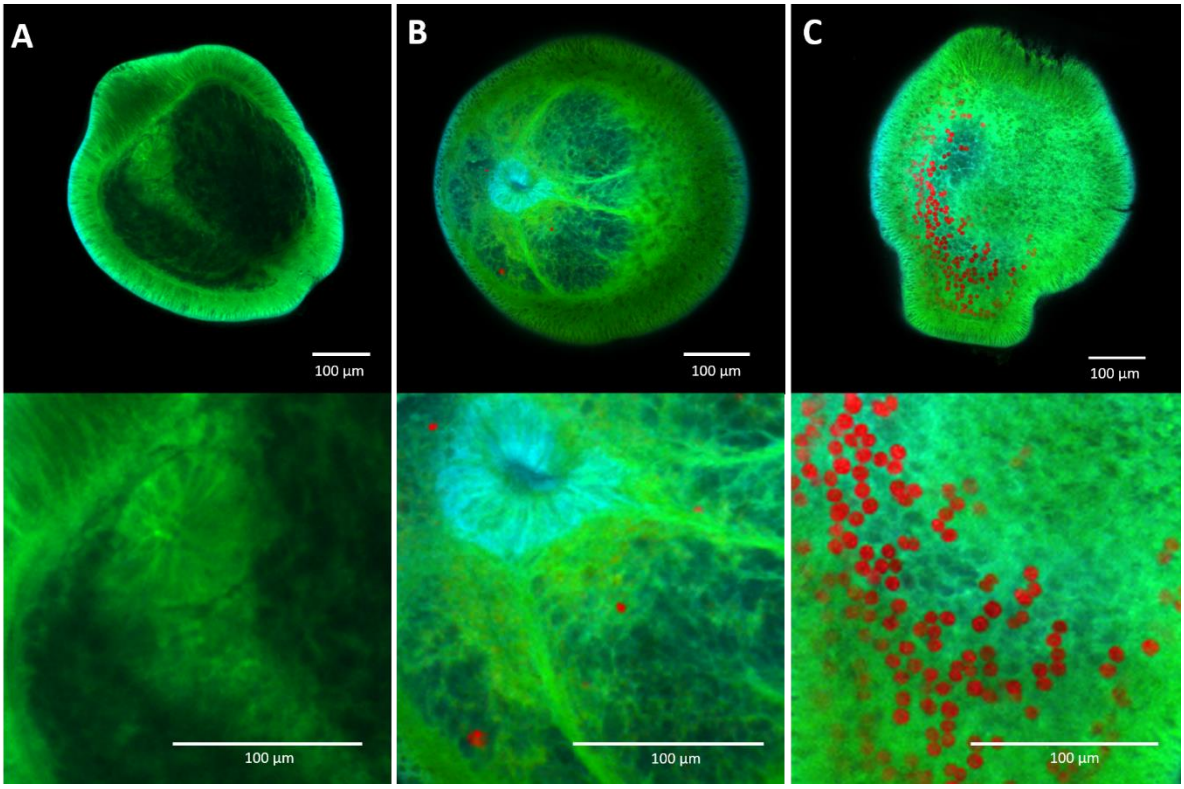

Figure 5.6. Confocal microscopy images of aposymbiotic *Acropora millepora* larvae (A), larvae containing chromerid cells (B) and larvae containing *Cladocopium goreaui* cells (C), 14 days post microalgal introduction to the larvae. Red pigmentation represents the chromerid and *C. goreaui* cells, while larval autofluorescence is represented in green.

5.5 Discussion

The thermal and herbicide tolerances of the four chromerid strains was highly variable and differed substantially to that of the known photosymbiont of corals, *Cladocopium goreaui.* Three of the four chromerid strains exhibited high thermal tolerances and two strains had exceptional herbicide tolerances, greater than observed for other photosynthetic microalgae (e.g. Jones et al. 2003; Magnusson et al. 2010; van Dam et al. 2015; Mercurio et al. 2016). Although coral larvae were able to take up each of the four chromerid strains, colonisation was low (less than 8 algal cells *per* larva) in comparison to that of *C. goreaui* (over 600 algal cells *per* larva).

The effect of temperature on free-living microalgal photochemistry

Photophysiological traits were highly variable between the microalgal strains under warming conditions. *Cladocopium goreaui* was the most sensitive, with negative effects of increasing temperature that were particularly severe with longer exposure. The impairment of photosynthetic function in the Symbiodiniaceae with elevated temperature is well-documented, often occurring above 30 °C (e.g. Coles and Jokiel, 1977; Hill et al., 2004; Iglesias-Prieto et al., 1992; Takahashi et al., 2009) and has previously been shown in this same isolate in **Chapter 2**. The photosynthetic

apparatus is susceptible to heat stress. In the Symbiodiniaceae moderate heat stress can result in photoinhibition of the chlorophyll *a* and *c* containing protein complex, photosystem II (PSII) (Warner et al. 1999a), whereby heat (combined with light) disrupts the electron transport system (Jones et al. 1998) through several proposed mechanisms. These include the production of highly reactive singlet oxygen produced by PSII, that in turn causes further disruption to the electron transport system, photosynthetic pigments, proteins and thylakoid membranes (Hideg et al. 1994; Telfer et al. 1999; Niyogi 1999; Krieger-Liszkay 2004). Photoinhibition occurs when the rate of photodamage to PSII exceeds the rate of its repair (Takahashi et al. 2004; Warner et al. 1999). The dramatic drop in photosynthetic efficiencies and the increase in maximum pressure over PSII (Qm) observed for *C. goreaui* indicates severe photoinhibition for temperatures above 30 °C in this study.

The two strains least affected by elevated temperature were the two *Chromera velia* strains, *C. velia1* and *C. velia2*, where only small decreases in photosynthetic efficiencies were observed after 22 days at 32 °C. Photosynthesis in *C. velia* has been described as highly efficient despite representing a basic system, i.e., a simple composition of pigments that includes only chlorophyll (Chl) *a*, violaxanthin and a novel isofucoxanthin-like carotenoid (as major components) and a lack of any accessory pigments (Moore et al. 2008). Violaxanthin has been shown to act as a key factor in efficient photoprotection in *C. velia* through the unusually fast de-epoxidation of vioxlaxanthin (Kotabová et al. 2011). In agreement, increases in violaxanthin in *C. velia* (isolate from Moore et al., 2008) grown under high light conditions resulted in a doubling in non-photochemical quenching values, reflecting the dissipation of non-photochemical energy, and 30 % reduction in excitation pressure over PSII compared to low light conditions (Quigg et al. 2012). These mechanisms may have occurred in *C. velia1* and *C. velia2* as a thermal acclimation response in my experiments, which is supported by the stable maximum pressure over PSII (Qm) across increasing temperature conditions.

Vitrella brassicaformis as well as the unknown chromerid, had lower photosynthetic efficiencies at the control 27 °C compared to the two *C. velia* strains and *C. goreaui* and exhibited photophysiologies most similar to each other. Unlike the two *C. velia* isolates, *V. brassicaformis* was negatively affected by temperature, although low quantum yield values and pressure over PSII values stabilised after 22 days. *Vitrella brassicaformis* and *C. velia*, although closely related, differ in several features that could underpin their differences in photochemistry and response to elevated temperature. For example, *Chromera* spp. produce isofucoxanthin while *Vitrella* spp. rely on vaucheriaxanthin (Moore et al. 2008; Oborník et al. 2012). While the roles of *V. brassicaformis*

pigments are unknown, such differences could result in variations in light harvesting and non-photochemical quenching capacity. Morphologically, *V. brassicaformis* possesses a multiple-laminated cell wall and a large conspicuous pyrenoid that are absent in *C. velia* (Oborník et al. 2012, Appendix 5.2). Although the cell wall layers are transparent, it is possible that the amount of light reaching the chloroplast is reduced. A large pyrenoid is present in cells of *V. brassicaformis* but not in *C. velia.* Pyrenoids are associated with the operation of a carbon concentrating mechanism (Giordano et al. 2005) and perhaps their presence in *V. brassicaformis* could account for differences in photosynthetic efficiencies through differences in carbon fixation. Finally, since the photosynthetic complexes are found within the thylakoid membrane in the chloroplast, changes in its composition under increased temperature may play a role in the thermal stability and/or acclimation of the light harvesting complexes (Tchernov et al. 2004). Specifically, galactolipids play an important role in thylakoid membrane stability (Wada and Murata 2009) and mass spectrometry and transcriptomic analyses have shown that *C. velia* and *V. brassicaformis* differ in their galactolipid content (Khadka et al. 2014). This may result in differences in the stability of the photosynthetic membranes that could account for some of the observed differences in thermal tolerances between microalgal species.

The effect of diuron and diuron with elevated temperature on free-living microalgal photochemistry

Cladocopium goreaui was the most sensitive microalgal strain to diuron requiring 7.4 µg/L at the control temperature of 27 °C for the inhibition of the effective quantum yield by 50 % (EC50) while at 32 °C the photosynthetic efficiency was already more than 50% inhibited before any diuron additions. Previous reported EC50 values testing the potency of diuron on free-living members of the Symbiodiniaceae have varied from 1.1 µg/L (van Dam et al. 2015) to 5.5 µg/L (Jones et al. 2003). The former experiment showed a small increase in diuron sensitivity for two Symbiodiniaceae species with elevated temperature. Other tropical microalgae at ambient 24 - 26 °C have displayed EC50 values of between 2.1-4.4 µg/L (Magnusson et al. 2010; Mercurio et al. 2018). Although there is apparent variation in diuron sensitivity between tropical microalgae as well as between members of the Symbiodiniaceae, the concentration of diuron required to inhibit effective quantum yields by 50% are generally below 10 µg/L (but see Kottuparambil et al. (2013) where a cyanobacterium exhibited an EC50 of 7.15-14.8 µg/L) and similar in magnitude. The chromerids studied here are far more tolerant of diuron than *C. goreaui* and to my knowledge any other reported marine algae. The two strains of *C. velia, C. velia1* and *C. velia2*, had EC50 values of 30 and 22 µg/L, respectively, and while they became more diuron-sensitive with elevated temperature, they still had high EC50 values

of 17 and 18 µg/L, respectively. Despite their comparatively low sensitivity to diuron compared to *C. goreaui*, they were not able to match the unprecedented diuron tolerances of *V. brassicaformis* and the unknown chromerid that required 140-fold more diuron before the effective photosynthetic quantum yields (QYs) were inhibited by 50%, with EC50 values of between 971 and 1702 µg/L. Furthermore, while the effective QY of *V. brassicaformis* was inhibited by over 25% at 32 °C, before the addition of diuron, the extreme diuron tolerance of the unknown chromerid was enhanced by elevated temperature.

The mechanism behind diuron toxicity on photosynthesis is well understood. Diuron targets PSII in the chloroplasts of plants and algae by competing with the plastoquinone binding site of the D1 electron acceptor in PSII. This blocks the transfer of electrons, resulting in decreased photochemical efficiency (Van Rensen 1989). Although the mechanisms underlying the differences in diuron tolerances between algae are unknown, I suggest several possibilities. Firstly, high concentrations of diuron may result in chronic photoinhibition (Jones 2005) and algae differ in their abilities to combat photodamage through the rate of repair of their PSII reaction centres (Takahashi et al. 2004, 2009), as well as exhibiting differing abilities to combat the excessive production of reactive oxygen species that likely occur from a disruption in electron flow (Lesser, 1997; McGinty et al., 2012; Suggett et al., 2008; Wietheger, Starzak, Gould, & Davy, 2018). Secondly, the low photosynthetic efficiencies exhibited by *V. brassicaformis* and the unknown chromerid could result in a lower base level of reactive oxygen species production that could allow higher diuron (and temperature) tolerances here. Thirdly, differences in the binding site of the D1 protein could exist between the chromerids and other microalgae, as well as between species of chromerids, reducing or enhancing the effectiveness of diuron. It is possible that a mutation(s) on the psbA gene encoding the D1 protein could be a feature that explains both the diuron tolerance and lower photosynthetic efficiencies of *V. brassicaformis* and the unknown chromerid (without diuron) where photosynthesis is limited by the efficiency of electron binding and transfer in PSII. Finally, a plausible explanation as to the extraordinary diuron-tolerance of *V. brassicaformis* may simply result from their unique morphology. *V. brassicaformis* has up to a dozen cell walls layered upon each other (Oborník et al. 2012), which could act as a barrier against diuron. Indeed, Obernik and colleagues were unable to use DNA-staining dyes in their study on *V. brassicaformis*, due to the thickness and impermeability of the cell wall. This multi-layered cell wall could result in a trade-off between a reduced amount of light available for photosynthesis with protection against toxic chemicals, such as herbicides.

I observed that the unknown chromerid was less sensitive to diuron with elevated temperature, with a 61% increase in effective QY after 22 days of exposure to 32 °C. It is possible that the binding affinity of diuron is reduced at elevated temperature with this strain through the D1 protein rate of turnover and/or repair or due to conformational changes to the D1 protein binding site (Jones and Kerswell 2003). The culture history of this strain was different to the other chromerids, having spent 2.5 years growing under 30 µg/L of diuron. It is possible that over multiple generations, the unknown chromerid went through adaptive changes, that resulted in a lowered sensitivity to diuron and in parallel to elevated temperature; genetic correlations among traits can exist, whereby selection on one trait can elicit a response in another (Stanton et al. 2000; Etterson and Shaw 2001; Blows and Hoffmann 2005). Indeed, the photosynthetic apparatus that is the target of diuron is also sensitive to temperature and thus selection for herbicide tolerance may have correlated with increased thermal tolerance for the unknown chromerid.

The effect of temperature and diuron on coral larval uptake of microalgae

At least one cell of each microalgal species was observed within *Acropora tenuis* and *Acropora millepora* larval tissue during this experiment, which shows that the chromerids can enter and be hosted by the larvae. However, there was no apparent correlation between the temperature and diuron tolerance of the microalgal strains *in vitro* with their uptake by coral larvae or larval mortality in either coral species tested. At 27 °C, the greatest microalgal uptake was *Cladocopium goreaui* by both coral species.

Acropora tenuis was more successful at taking up the chromerids compared to *A. millepora*, which displayed minimal colonisation across all three temperatures and under diuron conditions. At 27 °C in the absence of diuron, *C. goreaui* by far had the greatest colonisation-ability with over 600 cells *per* larva. *C. velia1* and *C. velia2* were the next most taken-up by *A. tenuis* larvae, containing a mean of approximately six chromerid cells. These results are similar to those found by Cumbo and colleagues, where short-term inoculation (up to three days) for *A. digitifera* and *A. tenuis* larvae resulted in fewer than 10 *C. velia* cells *per* larva, compared to over 100 cells of *C. goreaui* per larva (Cumbo et al. 2013). In my study, the uptake of *V. brassicaformis* and the unknown chromerid was minimal. The low photosynthetic efficiencies observed for *V. brassicaformis* and the unknown chromerid *in vitro* could be a factor for their unfavourable symbiosis with coral larvae, where algae are unable to meet the energy demands of the larvae. Conversely, the higher uptake by *A. tenuis* larvae of *C. velia1* and *C. velia2* could be, in part, due to their higher photosynthetic efficiencies (which are comparable to those for *C. goreaui*).

There has been debate as to where the chromerids may sit on the scale from parasitism to mutualism. Cumbo et al. (2013) indicated the potential for the chromerids to form a mutualistic relationship. These authors showed that three chromerid strains, including *C. velia1* and *C. velia2*, were located within the larval endoderm and ectoderm of corals, thus supporting a potentially mutualistic relationship. In contrast, Mohamed et al. (2018) investigated the transcriptomic response of *A. digitifera* larvae exposed to a different *C. velia* strain (Moore et al. 2008) up to 48 h after their introduction and concluded that *Chromera* is not a mutualist due to the transcriptomic resemblance of the coral larvae to a typical host-response to parasites or pathogens. These studies, along with ours, remain inconclusive as to the symbiotic relationship that the chromerids may have with corals. In my study, larval mortality was not significantly increased in the presence of chromerids, compared to *C. goreaui*. Furthermore, I did not observe increased uptake by the chromerids under elevated stress conditions (elevated temperature and/or the presence of diuron) that might have been indicative of a parasitic relationship.

Although larval uptake of microalgae was generally lowered in the presence of diuron, here was one major exception. *A. tenuis* hosted a significantly greater number of *C. goreaui* cells at 30 °C in the presence of diuron, compared to no diuron. This result was unexpected as *C. goreaui* was the most sensitive algal strain to diuron *in vitro*, and these observations were not mirrored in *A. millepora* nor any previous studies on coral-algal symbioses under diuron conditions. The literature supports a negative effect of diuron on the coral-Symbiodiniaceae symbiosis. For example, significant reductions in effective QY were recorded at very low diuron concentrations (≥ 1 µg/L) across four GBR coral species, while higher diuron concentrations (10 µg/L and above) resulted in the significant loss of symbionts, and tissue retraction, causing coral bleaching (Jones et al. 2003; Cantin et al. 2007). The expulsion of symbionts from GBR coral *Pocillopora damicornis* was observed at 10 µg/L of diuron in both recruits and adult colonies (Negri et al. 2005). Generally, a detrimental and mostly additive effect of elevated temperature combined with diuron on corals and other marine organisms has been documented (Negri et al. 2011; van Dam et al. 2012, 2015; Wilkinson et al. 2017). However, Jones and Kerswell (2003) found that diuron phytotoxicity of Symbiodiniceae within the coral *Seriatopora hystrix* was less at 30 °C than 20 °C and the toxicity of diuron appears to increase on either side of the thermal optimum for marine species (Negri et al., 2011 and Wilkinson et al., 2017).

Potential for the chromerids as alternative symbionts under stressful conditions

I found a highly variable response of free-living Symbiodiniaceae and chromerids to elevated temperature and diuron. The two *C. velia* strains showed comparatively high and sustained thermal tolerance and were moderately diuron-tolerant, while the *V. brassicaformis* strain exhibited high levels of diuron tolerance but was thermally sensitive. The unknown chromerid exhibited both thermal tolerance and an extreme diuron tolerance, with diuron being less toxic at elevated temperature. The photosynthetic advantages that the chromerids exhibited under stress *in vitro* did not affect the onset of symbiosis; larval uptake of algal cells was low regardless of temperature or diuron treatment. *Cladocopium goreaui* remained the microalgal strain most able to colonise both coral larval species. Thus, my results do not support the chromerids being dominant symbionts of the coral species tested. Given the low densities of the chromerids in the coral larvae, it is unlikely they contribute significantly to the nutrition of their coral host and my results suggest there is no value in the use of the chromerids as alternative/additional symbionts in an assisted evolution approach. However, it is possible that it takes longer than 14 days for corals to establish symbiosis with chromerids compared to Symbiodiniaceae. The previous two experiments investigating coral larval uptake of the chromerids only lasted 3 days (Cumbo et al. 2013) and 24 hours (Amin R Mohamed et al., 2018), the latter of which did not quantitively measure algal uptake. Thus, it is unknown whether a longer duration of observation would result in greater *in hospite* densities. It is also possible that, like the Symbiodiniaceae, there is host-specificity in the symbioses with the chromerids. Indeed Cumbo et al. (2013) showed that *A. tenuis* larvae exposed to three strains of *C. velia* (including Mdig2 and Mdig3 used here) contained approximately six Mdig3 cells *per* larva, compared *C. goreaui* 160 cells *per* larva after three days post-introduction; results that are in line with *A. tenuis* uptake in this study, after 14 days. In contrast a different coral species, *A. digitifera,* hosted Mdig3 in numbers that exceeded the *in hospite* algal cell densities of *C. goreaui* after both 24 hours and 2 days (Cumbo et al. 2013).

Finally, it is possible that a symbiont that may benefit one life stage of a coral, may not benefit another as the nutritional needs of corals are different throughout their life-history (Abrego et al., 2008; A. M. Jones et al., 2008; Quigley et al., 2016). All strains of chromerids so far have been isolated from adult coral colonies and it is possible that the uptake of the chromerids is unfavourable during the early life stages of corals. Indeed unlike adult corals, no apicomplexan DNA was detected among planulae from broadcast spawning coral species, however planulae from brooding coral species tested positive for apicomplexan DNA (Kirk et al. 2013a). I recommend future experiments

should investigate uptake, symbiosis establishment and consequences of the chromerids across coral life stages and different species.

The differences in morphology, ultrastructure and genetics that have already been reported between *C. velia* and *V. brassicaformis* in the literature is large despite being represented by only a very small handful of isolates, with only one isolate of *V. brassicaformis* having been described (Obornik et al. 2012). Extensive phenotypic and physiological variation also exists within the Symbiodiniaceae, between genetically distinct species as well as within species (Parkinson et al. 2016; Suggett et al. 2017; Swain et al. 2017). This intra-species variation, along with the range of photophysiological responses to stressors that I observed in this study, indicates that with further discovery of chromerid strains there is likely additional diversity to be characterised. It is possible that there are other species within the phylum Chromerida that may function better as coral symbionts than those studied here.

Conclusions

From an assisted evolution stand point, this study has identified highly temperature tolerant and/or herbicide resistant microalgae that are found associated with corals and that can enter the coral host, albeit minimal. The Symbiodiniaceae symbiosis is in jeopardy in an era of rapid ocean warming and increased local stressors and understanding of the genetic architecture underpinning high stress tolerances in the chromerids may inform genetic engineering approaches targeted at enhancing thermal tolerance of Symbiodiniaceae symbionts. The insertion and expression of a new gene into the marine microalga *Chlorella* has been successfully used to achieve the expression of heterologous proteins for various applications (Yang et al. 2016). Such genetic manipulations may be applied to coral symbionts with the aim of enhancing their thermal tolerance.

Further studies should be carried out to isolate and characterise additional strains of the chromerids, and to investigate their relative ability to colonise all life stages of coral, their potential benefit to the coral host, and the cellular and genetic mechanisms that underpin any stress responses of these elusive algae.

Chapter 6: General Discussion

Coral reefs are under threat from a rapidly warming planet. Pan-tropical losses of coral cover are becoming more frequent (Hughes et al. 2018). Anthropogenic CO_2 emissions that are driving global warming are unlikely to be curbed and thus the need for direct human intervention may be necessary to ensure the persistence of coral reefs. Investigation into the assisted evolution of the coral holobiont is underway in an effort to enhance the resilience of corals. This thesis explores the manipulation of the coral's algal photosymbionts to understand and enhance their environmental tolerance and the resilience of the holobiont to climate-related environmental change. I found that laboratory directed evolution can be used to enhance the thermal tolerance of the free-living Symbiodiniaceae (**chapters 2 and 4**),this is reflected in transcriptome-wide differences in gene expression (**chapter 3**). However, the one selected Symbiodiniaceae symbiont strain that I tested *in hospite* conferred limited benefits to the thermal tolerance of coral holobionts (**chapter 2**). Furthermore, I found that chromerid microalgae widely vary in their environmental tolerances but are unlikely to be dominant symbionts of corals (**chapter 5**).

Laboratory thermal selection in the Symbiodiniaceae

After 2.5 years of culture at 31 °C the common generalist Symbiodiniaceae species *Cladocopium goreaui* exhibited photochemical traits comparable to the control (wild-type at ambient temperature) and showed a positive growth rate (**chapter 2**). In **chapter 4**, I demonstrate that three more cultures of the Symbiodiniaceae belonging to different genera, selected for one year to elevated temperature conditions, had superior growth rates compared to their counterpart wild-type cells under elevated temperatures.

Previous studies using experimental evolution techniques on other microalgae have focused on phytoplankton such as other dinoflagellates, diatoms and coccolithophores (e.g. Flores-Moya et al., 2008, 2012; Huertas et al., 2011; Schlüter et al., 2014). This earlier work showed that it takes a minimum of 250 generations before the fitness of selected algal cells under heat stress can exceed that of the wild-type cells under heat stress. For example, the dinoflagellate *Procentrum triestnum* exposed for 250 generations (two years) to elevated temperature and reduced pH conditions exhibited a 50-60% increase in growth rate compared to the wild-type cells under the same conditions (Flores-Moya et al., 2012). The coccolithophore *Emiliania huxleyi* exhibited a 16% increase in growth rate after 460 generations (1 year) at elevated temperature and pCO_2 conditions

(Schlüter et al. 2014). In **chapter 2** I show a stable adaptive response to 31 °C after ~80 generations (~ 2.5 years) for *C. goreaui* and in **chapter 4** I observed a stable adaptive change after only 41-69 generations (~ 1 year) for three other Symbiodiniaceae species. Such rapid adaptation could be attributed to the slightly different selection methods that I used compared to most previous stduies. Following similar methods to (Huertas et al. 2011) I subjected replicate populations of each species to incremental increases in temperature but only to a level where positive growth was possible. This allowed the maintenance of large population sizes, maximising the number of random mutations that may occur, while also exerting a strong selection pressure. This technique also permitted an assessment of the maximum adaptive capacity of each species in question.

While four of six genetically distinct Symbiodiniaceae exhibited a stable adaptive change after being subjected to thermal selection in **chapters 2 and 4**, each varied in their maximum adaptive capacity. For example, populations of *Symbiodinium* were able to survive at the first selection temperature of 30 °C, populations of *Gerakladium* were able to grow at the second temperature of 31 °C and *Fugacium* at 34 °C. For each genus, at least one replicate population of thermally selected cells exhibited growth and/or photosynthetic efficiencies that exceeded their counterpart wild-type population transplanted into elevated temperature. These results show that rapid thermal adaptation is possible across multiple Symbiodiniaceae genera but that the maximum adaptive capacity varies within the Symbiodiniaceae. This has implications in terms of the choice of Symbiodiniaceae species for directed evolution efforts aimed at increasing the thermal tolerance of coral stock used for reef restoration. Furthermore, my results suggest that naturally heat-tolerant phenotypes/genotypes of the Symbiodiniaceae may already occur on the Great Barrier Reef, perhaps selected during a period of higher than usual temperature. While this is assuming that my selection regimes match environmental trajectories, such naturally occurring symbionts could be used in coral conservation efforts and are worth identifying and investigating.

I tested the effect of heat stress on corals that were colonised by the thermally selected *C. goreaui* in a second experiment in **chapter 2.** I found that corals harbouring the thermally selected symbionts bleached slower than those hosting the wild-type cells. The effect of coral recruits harbouring thermally tolerant algae was not as pronounced as I had expected, when the free-living selected *C. goreaui* exhibited a clear superior thermal tolerance compared to the wild-type cells. The host environment is different to the free-living environment where the Symbiodiniaceae may exist in a reduced metabolic state (Goiran et al. 1996) with host-controlled nutrient provisions (Falkowski et al. 1993; Stat et al. 2008) and it is possible that the influence of the host masked fitness benefits that

the symbiont acquired *ex hospite,* to some degree. In **Chapter 4** I show that replicate populations of selected cells exhibited differences in growth and photosynthetic efficiencies after long-term selection. Indeed, each replicate population is an independent evolutionary unit that may have evolved different mutations. Given the intra-specific variability between evolved lines, this suggests that there is high variability in the potential success rate of heat tolerant symbionts that could affect conservation efforts of selected populations *in hospite.* It is therefore worthwhile for many replicate populations (more than three) to be put through future evolutionary experiments . In addition, it is important to consider host factors that exert control over their algal symbionts such as through controlling the translocation of photosynthetically derived carbon and nitrogen cycling (Wang and Douglas 1997; Biel et al. 2007; Rädecker et al. 2015). It is likely that a combination of both symbiont and host-derived traits contribute to the success of a symbiosis, thus it will be important to find what symbiont and host traits would favour uptake and establishment of symbionts. **Chapters 2** and **4** also highlight the need to further test the effect of heat exposure on the coral-SS symbiosis across different life stages and different coral species. It is possible that symbiont growth may be an important trait to artificially select for when the goal is to enhance thermal tolerance in free-living Symbiodiniaceae*,* but it may not be an appropriate trait to select for if the aim is to augment coral bleaching tolerance. This is an important avenue for future research.

Genetic regulation of thermal tolerance in heat-selected Symbiodiniaceae

Chapter 3 examines the genetic pathways underpinning the phenotypic response to elevated temperature of selected and wild-type *C. goreaui*. A significant difference in the transcriptome-wide responses between selected and wild-type cells was clear, with many genes differentially expressed at a fold-change as high as >8. While I expected significant differences in gene expression based on the divergent phenotypic differences observed by the wild-type and selected cells under heat stress in **chapter 2**, this result contrasted with many of the past comparative transcriptomic studies on the Symbiodiniaceae and their response to changes in environment. For example, some Symbiodiniaceae studies exhibited a less than 2-fold difference in gene expression (Leggat et al. 2011; Gierz et al. 2017) or no changes (Barshis et al. 2014). Other dinoflagellates have shown similarly low levels of differential gene expression (Okamoto and Hastings 2003; Van Dolah et al. 2007). Only one earlier Symbiodiniaceae study has shown levels of differential expression in line with those I observed in **chapter 3;** notably, this study was conducted on two Symbiodiniaceae populations, one of which was the the heterogenous *C. goreaui* culture from which the wild-type culture I used was derived from a single isolated cell (Levin et al. 2016). Based on results from the latter study and **chapter 3**, it is possible that the ability to exhibit large, plastic changes in gene

expression are characteristic of *C. goreaui* and could point to the relatively generalist nature of this species, as well as its dominance across many GBR corals. Indeed, translational regulation and post-translational modifications have been suggested to be the major factor in driving changes in protein expression under heat stress (Barshis et al. 2014) and thus their regulation could be the dominant response to environmental change used by other species of the Symbiodiniaceae. Many more comparative transcriptomic studies on a wide range of Symbiodiniaceae species should be carried out to understand the variation in species-specific responses to heat stress, as well as simultaneous multiple stressors, while measuring various traits. Furthermore, such studies should link the *in vitro* responses of the Symbiodiniaceae with their responses to environmental change *in hospite*.

Wild-type cells at elevated temperature exhibited a classic stress response and after 28 days of heating, their tolerance limits were exceeded. This result was not unexpected based on the physiological decline of the WT@31 cells over time in **chapter 2**. Photosynthetic genes were consistently downregulated while genes and pathways involved in chaperonin activity, protein degradation, protection against DNA damage, fatty acid metabolism and transport and programmed cell death were upregulated in a classic stress response. Programmed cell death has been observed before in the Symbiodiniaceae and their hosts under heat stress (e.g. Desalvo et al., 2008; Mohamed et al., 2016; Zhou, Liu, Wang, Luo, & Li, 2019) and is probably triggered as a last resort to sacrifice damaged cells to save others (Arnoult et al. 2002; Huettenbrenner et al. 2003; Dunn et al. 2004). By the end of the experiment, after 35 days of heating, there was an order of magnitude decrease in DEGs compared to 28 days of heating, suggesting the mean tolerance of the population was exceeded and transcriptome-wide shut down. These results were in line with the physiological decline observed in **chapter 2** where WT cells at 31 °C were dying and there was an inability to detect photosynthetic traits within the population as cells became less viable. It is important to note, however, that in the context of a reducing population size, the transcriptomic response of the population could represent a high contribution by a relatively low number of viable cells. Instead of bulk population analyses, future transcriptomic experiments would be useful on single symbiont cells, given the high variation that I observed just between replicates of a population.

Conversely, selected cells exhibited a consistent transcriptomic response over time in response to elevated temperature. Similar genes and numbers of genes were up and downregulated, compared to the control, over time. Most notably, transcription factor activity was upregulated, while many typical stress response genes were downregulated. The sustained regulation of these genes may be vital to allowing the SS@31 to cope with elevated temperature, further supported by the inability of

the wild-type cells at elevated temperature to consistently upregulate transcription factor activity genes. Indeed, transcription factor activities have been discussed as genetic manipulation tools tool in crops to enhance stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2006; Agarwal et al. 2006; Lata and Prasad 2011) and such genes could be important targets for enhancing thermal tolerance in the Symbiodiniaceae.

A gene homologous to the type II secretion system was among of the five most upregulated genes by the selected cells at elevated temperature. This gene aligned with a 40 % and 50 % similarity to the published *Cladocopium* genome and to bacterial genomes, respectively, and it is therefore unclear whether it is encoded by the alga or by a closely associated bacterium. It's putative function in bacteria to secrete proteins such as toxins, proteases and lipases into the surrounding environment could be a key process for thermally selected Symbiodiniaceae in an attempt to remove damaging/damaged products of heat stress into the surrounding environment. Alternatively, it is possible that this transcript belongs to bacteria that reside in the same culture as the selected cells. If so, such bacteria could be important factors in the successful growth and photosynthesis of thermally selected Symbiodiniaceae. Indeed, in some cases, bacteria have been found necessary for the successful growth and survivorship of the Symbiodiniaceae and other dinoflagellates (e.g. Alavi, Miller, Erlandson, Schneider, & Belas, 2001; Ritchie, 2012). Further research should characterise the bacteria that are closely associated with the Symbiodiniaceae through techniques such as the growth of washed Symbiodiniaceae cells on agar and subsequent genome sequencing of pure bacterial cultures. Resulting genomes can be searched for the T2SS gene in question and cytogenic techniques such as Fluorescence *in situ* Hybridisation (FISH) can be used find if bacteria occur on the inside or outside of the Symbiodiniaceae cell. Alternative approaches could involve directly sequencing bacterial genomes isolated from washed Symbiodiniaceae cells with single cell genomics methods. If identified, this would allow informative microbiome manipulation experiments to determine the role of these bacteria in coral function and thermal resilience.

Downregulated genes in the selected cells included those involved in metabolism, lamellipodium and filopodium assembly and organisation, cell migration and motility. These genes were likely downregulated to conserve energy in a hotter environment. The downregulation of such genes could come at a cost for the coral-algal symbiosis. For example, a reduced metabolism at elevated temperature could result in changes in the nutrient exchange between the symbiont and coral partner which has been suggested to play a role in the breakdown of the symbiosis (Wooldridge 2009b, a; Wiedenmann et al. 2013; Pogoreutz et al. 2017). This could have been a reason why in

chapter 2, coral recruits colonised with the selected cells still bleached. Secondly, decreased cell motility could negatively affect Symbiodiniaceae dispersal, movement within the water column and thus the potential of encountering a coral host and the onset of symbiosis. Such trade-offs are important to consider in assisted evolution approaches.

Environmental tolerances of alternate coral-associated microalgae

In addition to the Symbiodiniaceae, other microalgae such as the Chromerida are associated with corals. **Chapter 5** found that the chromerids vary widely in their tolerances to warming and diuron exposure. Two populations of *Chromera velia* showed high and sustained thermal tolerance and were moderately diuron-tolerant, a population of *Vitrella brassicaformis* showed high levels of diuron tolerance but was thermally sensitive and an unknown chromerid population that had been cultured for 2.5 years under diuron selection exhibited both thermal tolerance and an extreme diuron tolerance. A number of reasons could explain the variation in the chromerid responses. For example, for *C. velia,* efficient photoprotection mechanisms based on differences in the composition of pigments compared to the Symbiodiniaceae (Moore et al. 2008; Kotabová et al. 2011; Quigg et al. 2012) could explain the stable photosynthetic efficiencies at elevated temperature observed. For *V. brassicaformis* differences in morphology may explain the exceptional diuron tolerance of the population studied in **chapter 5**; *V. brassicaformis* was found to have up to a dozen cell walls layered upon each other (Oborník et al. 2012) that could act as a barrier against diuron.

While the Symbiodiniaceae are the most common photosymbiont of corals, chromerids also contain a functioning photosynthetic plastid, raising the question as to whether they can act as alternate or additional symbionts of corals. In **chapter 5**, I found that coral larvae of two acroporid coral species were able to host the chromerids but in minimal numbers compared to *C. goreaui*. My results therefore do not support the chromerids as important symbionts of corals. This work is consistent with the only two other studies that have examined coral-chromerid interactions. Firstly, Cumbo et al. (2013) showed that acroporid coral larvae hosted *C. velia* at low densities after three days of exposure, at numbers that were comparable to my results after 14 days post introduction in **chapter 5**. Secondly, a comparative transcriptomic study observed a typical parasitic response of coral larvae exposed to *C. velia* compared to Symbiodiniaceae cells. Given the low chromerid cell densities that were hosted by the coral larvae it is unlikely that they contributed to the coral larvae's energy requirements. It is possible, however, that the chromerids function as symbiont in the later life stages of corals, which has not been tested.

Future research priorities

The enhanced thermal tolerance of the selected Symbiodiniaceae did not significantly improve holobiont bleaching tolerance under thermal stress (**chapter 2**). In addition, thermal and herbicide tolerances displayed by some chromerid populations did not affect their uptake by corals under environmental stress (**chapter 5**). Both experiments were carried out using the early life stages of corals, i.e., larvae and young recruits. It is possible that a symbiont that may benefit one life stage of a coral may not benefit another, as the nutritional needs of corals are different throughout their life history (Jones et al. 2008; Abrego et al. 2008; Quigley et al. 2016). All strains of chromerids so far have been isolated from adult coral colonies and their functional association with the early life stages of coral is unknown. Following on from these chapters, I recommend future experiments investigate uptake, symbiosis establishment and consequences of the thermally selected Symbiodiniaceae and environmentally resilient chromerids across coral life stages and different coral species. Furthermore, for the Symbiodiniaceae I have only tested one evolved lineage *in hospite* (**chapter 2**), where I chose to experiment with a randomly chosen evolved population of *C. goreaui.* However, it is clear from **chapter 4** that replicate populations of the same culture can independently evolve to exhibit varying degrees of fitness *in vitro*, suggesting that *in hospite* fitness differences among replicate selected cultures also exist. I propose that future experiments test the *in hospite* effect of multiple evolved lineages derived from the same clonal wild-type strain as well as to investigate why they might be variable despite originating from the same population.

Comparative transcriptomic studies such as in **chapter 3** can identify genes and functional pathways that underly thermal tolerance in heat-resistant symbionts. Such studies are important to inform genetic engineering studies targeting particular genes, or suites of genes, that aim to augment symbiont environmental tolerances, without having to carry out non-targeted and potentially lengthy evolutionary experiments (Murray et al. 2016). Importantly, such genetic studies should be carried out on Symbiodiniaceae strains that do confer thermal tolerance to the symbiosis. Indeed, laboratory evolutionary experiments that expose free-living microalgae for many generations to a selection pressure as in **chapters 2** and **4** ultimately select for faster growers and fast growth rates under elevated temperature *in hospite* may not be beneficial to the coral host, where the host acts to control and restrict symbiont number *in hospite*. Future directed evolution experiments should focus not only on growth rates of the Symbiodiniaceae at elevated temperatures but should explore which traits make them favourable in a symbiosis and use these as criteria for further selection. Comparative transcriptomic studies are informative in this case and should incorporate the symbiont and coral host transcriptomic response to environmental change to understand the underlying

mechanisms that are important for a successful symbiosis under heat stress. Having said this, free-living Symbiodiniaceae that make up the environmental pool of algae are crucial for horizontal uptake by corals and it is therefore still important to continue investigation into the Symbiodiniaceae *ex hospite*.

Concluding remarks

This thesis presents the first work into the assisted evolution of coral photosymbionts to adverse environmental conditions. Importantly, I have shown that thermal adaptation can occur rapidly across multiple Symbiodiniaceae species under laboratory thermal selection. However, the temperature-selected *C. goreaui* strain tested *in hospite* in **chapter 2** did not increase the thermal tolerance of the coral holobiont to an ecologically relevant level. It is therefore important to examine the *in hospite* performance of additional, thermally selected lineages with the same *in vitro* phenotype, as some may be able to increase thermal tolerance of the coral holobiont potentially due to different mutation(s) having independently occurred. Furthermore, some of the lab-evolved populations presented in **chapter 4** may be able to increase thermal tolerance of the holobiont, an assessment of which is a research priority. This thesis adds to a growing body of work that investigates direct intervention techniques for the coral and their associated microbes in a rapidly warming ocean. From the pre-conditioning of corals to adverse conditions to coral hybridisation and the manipulation of other coral microbes in a probiotic approach (van Oppen et al. 2015, 2017). With future research, the manipulation of coral photosymbionts has the potential to be used as a tool to aid corals persistence in conservation and restoration initiatives.

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Appendices

Appendix 3.1 Comparing raw Trinity transcriptome assemblies with processed transcriptomes for those of the wild-type (WT) *Cladocopium goreaui*, thermally selected (SS) *C. goreaui* and combined (WT and SS) transcriptomes for *de novo* raw Trinity assembly and processed transcripts where redundant transcripts were collapsed into the longest representative transcript (99% sequence similarity over 99% of the shorter transcript) using cd-hit-est

		Raw assembly	Representative sequences
WT	Total trinity "genes"	93,954	93,929
	Total Trinity "transcripts"	205,370	162,225
	Percent GC	52.82	52.49
	Mean length	1,190.55	1,087.97
	Complete BUSCOs		278 (91.8%)
ST	Total trinity "genes"	93,103	93,074
	Total Trinity "transcripts"	211,662	168,316
	Percent GC	53.3	52.57
	Complete BUSCOs		276 (91.1%)
	Mean length	1,237.75	1,216.06
WT+ST	Total trinity "genes"	124,243	124,187
	Total Trinity "transcripts"	287,352	266,077
	Percent GC	52.57	52.24
	Mean length	1,216	1,093
	Complete BUSCOs	238 (78.5%)	287 (94.7%)

Appendix 3.2. Numbers of up and down-regulated genes and number of replicates for the WT@31, SS@31, SS@27 compared to the WT@27 control (≥ 8-fold, FDR ≤ 0.001) across the three sampling points.

		WT@27					
		Day 21		Day 28		Day 35	
		Up	Down	Up	Down	Up	Down
WT@31		343	288	2689	105	192	12
		n = 1		n = 2		n = 1	
SS@31		186	686	296	279	339	274
		n = 2		n = 3		n = 3	
SS@27		236	142	976	142	77	90
		n = 3		n = 3		n = 3	

Appendix 3.3. log-ratio-mean-average (MA) plots with log fold-change (logFC) in gene expression and number of transcripts expressed (log counts) and volcano plots with log false discovery rates (logFDR) for genes expressed by the WT@31, SS@31 and SS@27 compared to the WT@27 control after 21 days during the experiment.

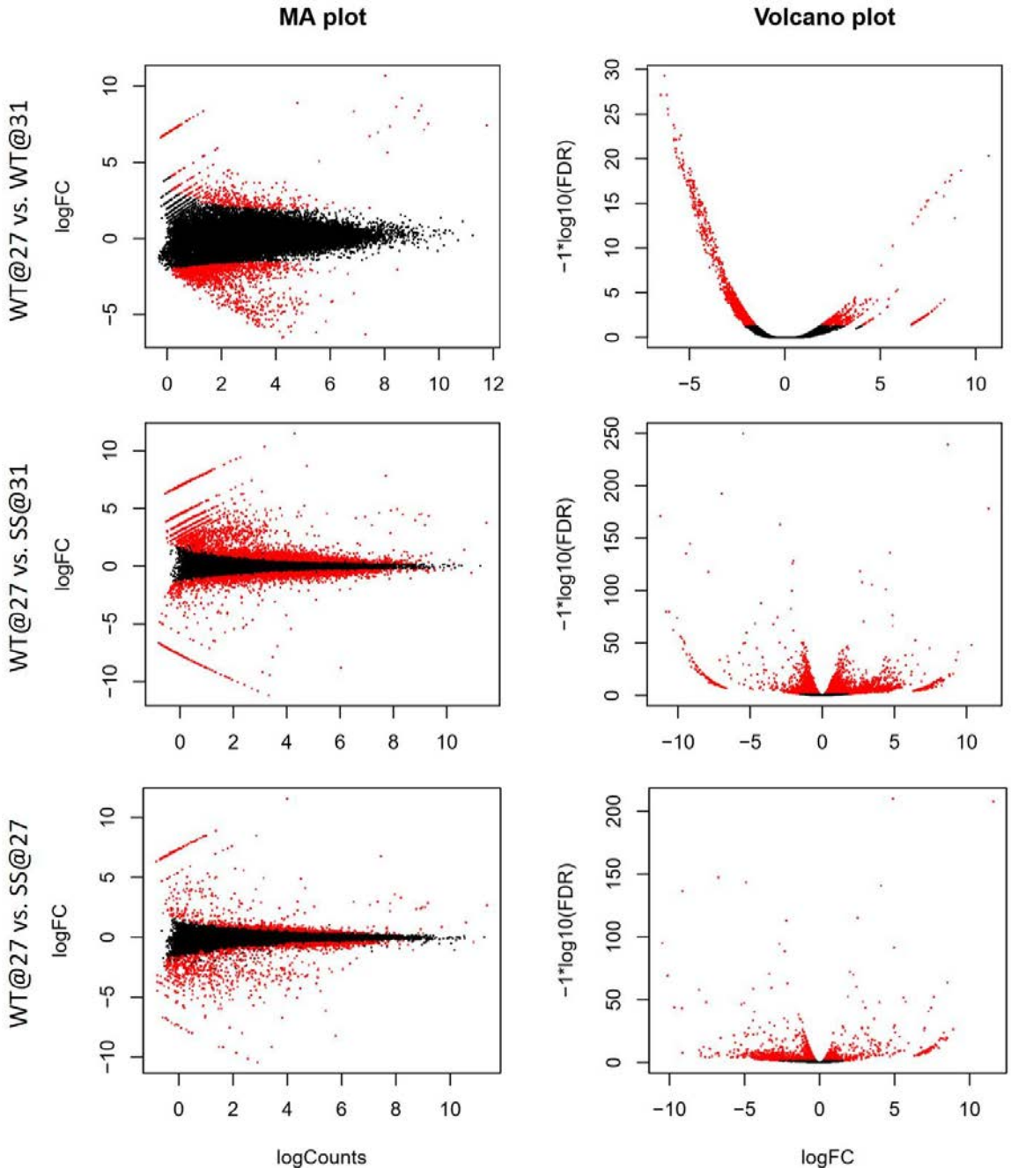
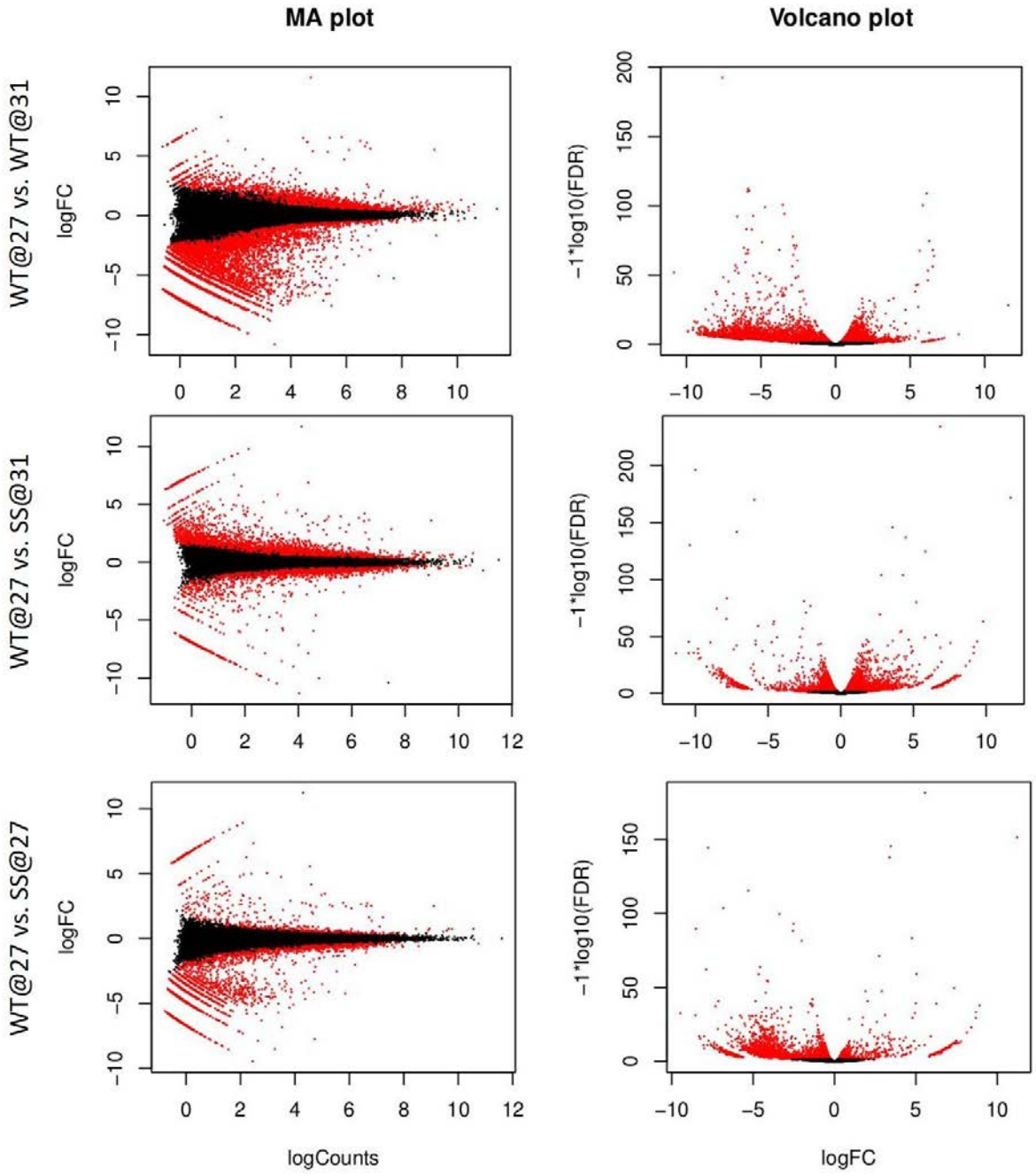
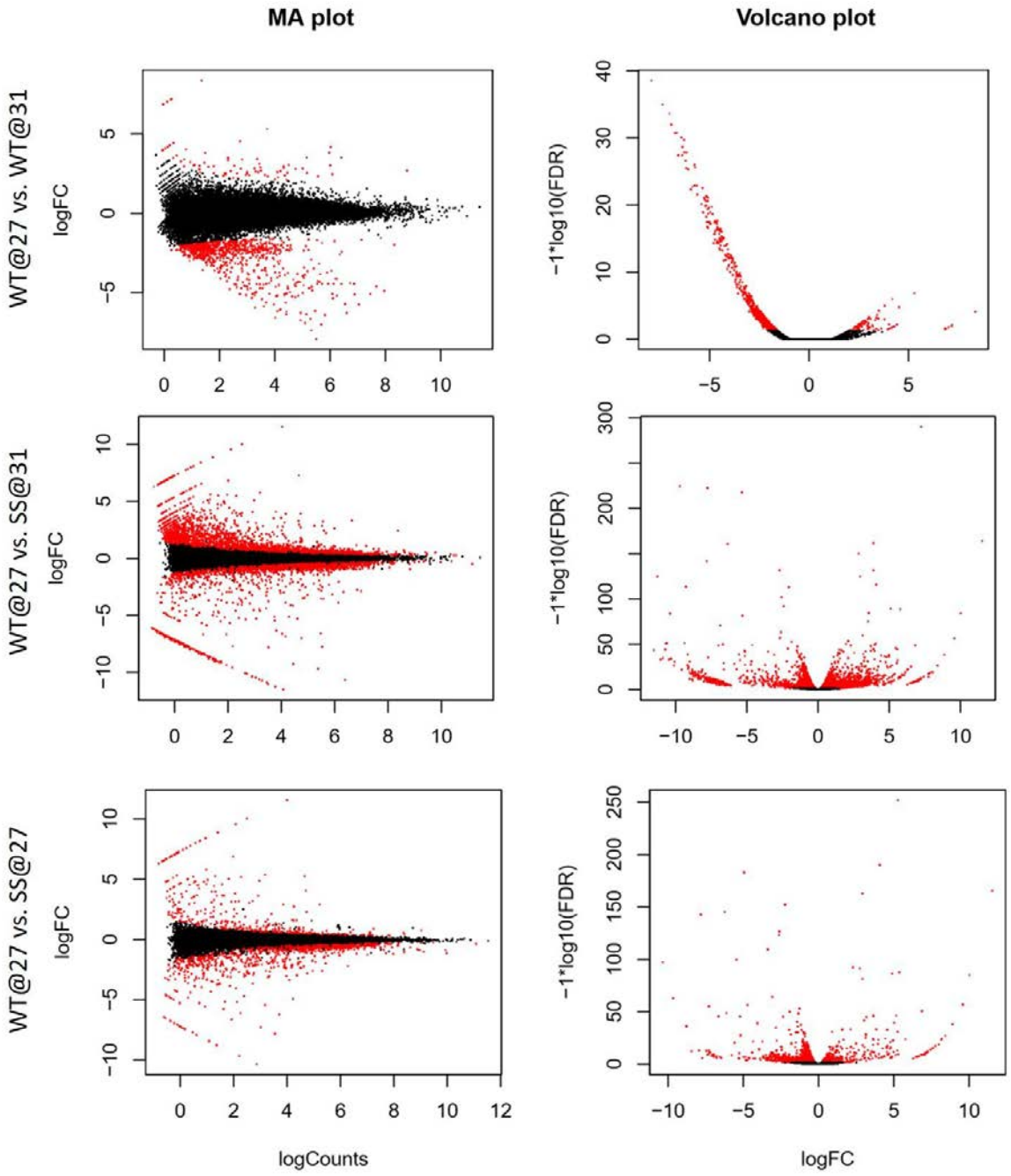


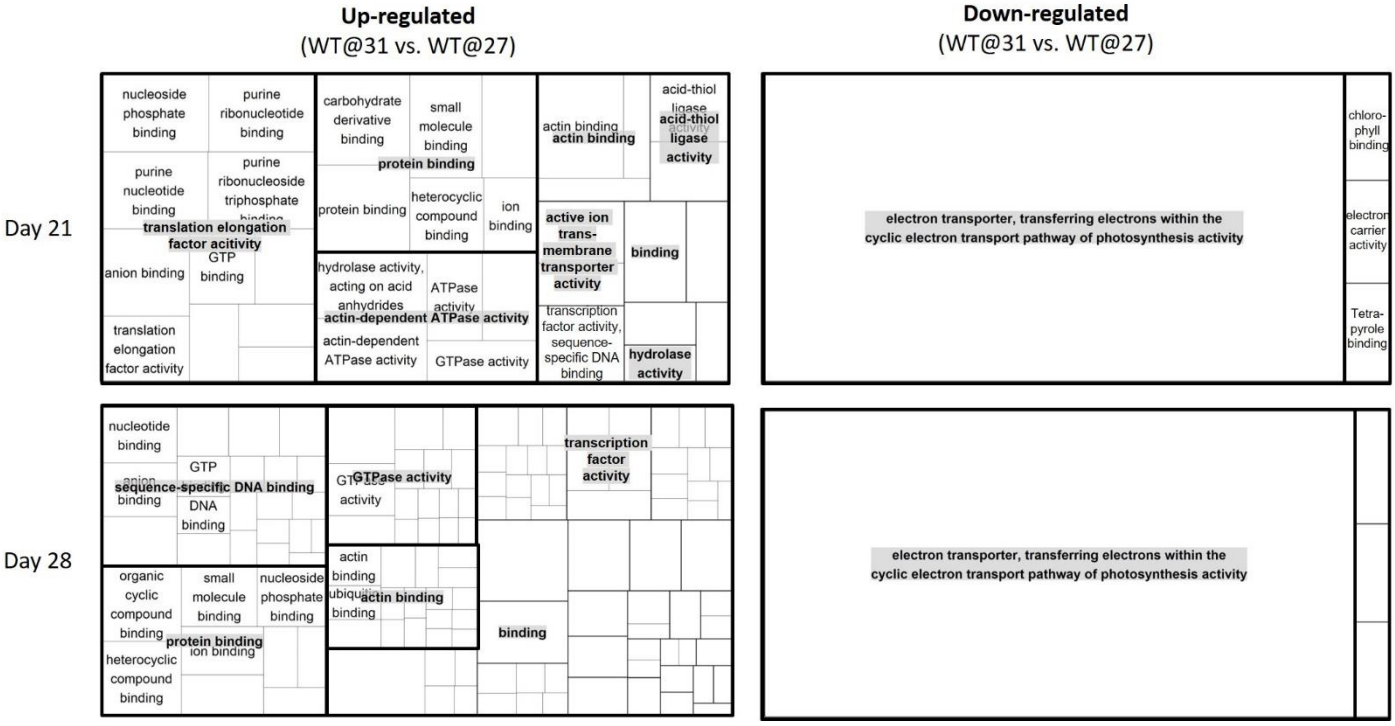
Figure 3.4. log-ratio-mean-average (MA) plots with log fold-change (logFC) in gene expression and number of transcripts expressed (log counts) and volcano plots with log false discovery rates (logFDR)) for genes expressed by the WT@31, SS@31 and SS@27 compared to the WT@27 control after 28 days during the experiment.



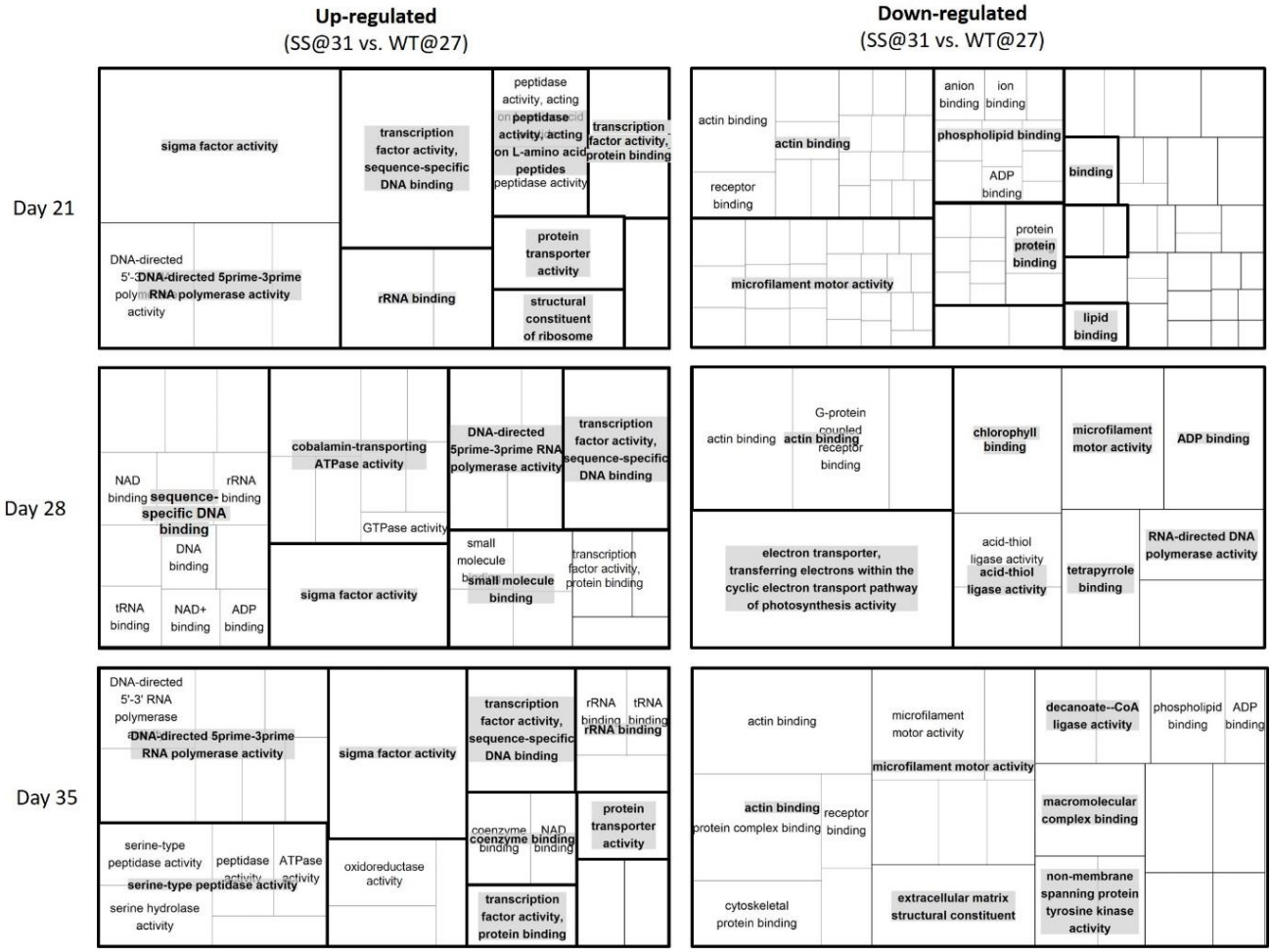
Appendix 3.5. log-ratio-mean-average (MA) plots with log fold-change (logFC) in gene expression and number of transcripts expressed (log counts) and volcano plots with log false discovery rates (logFDR) for genes expressed by the WT@31, SS@31 and SS@27 compared to the WT@27 control after 35 days during the experiment.



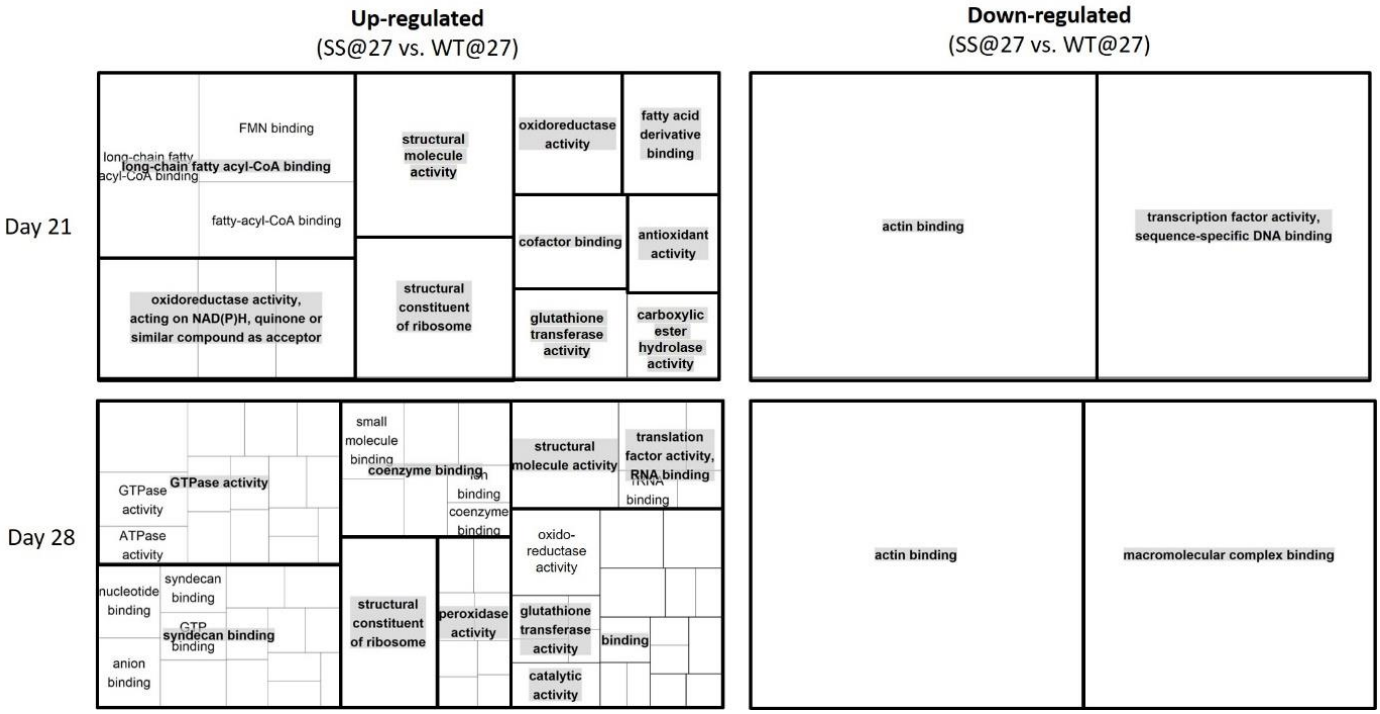
Appendix 3.6 Treemap of summarised molecular function Gene Ontology terms enriched for upregulated and downregulated biological processes for the WT@31 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Treemaps were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.



Appendix 3.7 Treemap of summarised molecular function Gene Ontology terms enriched for upregulated and downregulated biological processes for the SS@31 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Treemaps were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.



Appendix 3.8 Treemap of summarised molecular function Gene Ontology terms enriched for upregulated and downregulated biological processes for the ST@27 compared to the WT@27 control, across three time points. Each rectangle is a single cluster representative, joined into larger clusters of loosely related terms. Size of the rectangles reflect the p-value of the GO term in the GO analysis. Treemaps were created using REVIGO, based on a medium (0.7) allowed similarity with GO term sizes from the whole UniProt database and sematic similarity measure of SimRel.



Appendix 4.1 Statistically significant pairwise comparisons (Tukey's post-hoc tests) in mean growth rates (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark adapted maximum quantum yields (F_v/F_m) between *Symbiodinium* (ITS2 type A3c) populations during the 30°C reciprocal transplant experiment

			Pairwise comparison			
Trait	Day	ITS2 type	Population 1	Population 2	t-value	p-value
μ	17	A3c	SS@30a	WT@27	3.51	0.04
μ	17	A3c	SS@30a	WT@30	5.23	<0.01
μ	17	A3c	SS@30c	WT@30	4.60	0.01
$\Delta F/F_m'$	3	A3c	WT@27	SS@30a	4.54	<0.01
$\Delta F/F_m'$	3	A3c	WT@27	SS@30b	4.17	0.02
$\Delta F/F_m'$	3	A3c	WT@27	SS@30c	5.66	<0.01
$\Delta F/F_m'$	3	A3c	WT@30	WT@27	-6.23	<0.01
$\Delta F/F_m'$	10	A3c	WT@27	SS@30a	9.00	<0.01
$\Delta F/F_m'$	10	A3c	WT@27	SS@30b	8.11	<0.01
$\Delta F/F_m'$	10	A3c	WT@27	SS@30c	7.42	<0.01
$\Delta F/F_m'$	10	A3c	WT@30	WT@27	-7.42	<0.01
$\Delta F/F_m'$	17	A3c	WT@27	SS@30a	6.51	<0.01
$\Delta F/F_m'$	17	A3c	WT@27	SS@30b	7.92	<0.01
$\Delta F/F_m'$	17	A3c	WT@27	SS@30c	6.24	<0.01
$\Delta F/F_m'$	17	A3c	WT@30	WT@27	-8.71	<0.01
$\Delta F/F_m'$	26	A3c	WT@27	SS@30a	8.41	<0.01
$\Delta F/F_m'$	26	A3c	WT@30	SS@30a	-5.56	<0.01
$\Delta F/F_m'$	26	A3c	WT@30	SS@30b	-7.07	<0.01
$\Delta F/F_m'$	26	A3c	WT@27	SS@30b	8.95	<0.01
$\Delta F/F_m'$	26	A3c	WT@27	SS@30c	8.41	<0.01
$\Delta F/F_m'$	26	A3c	WT@30	SS@30c	-5.56	<0.01
$\Delta F/F_m'$	26	A3c	WT@30	WT@27	-13.97	<0.01
$\Delta F/F_m'$	3 and 26	A3c	WT@30	WT@30	-5.65	<0.01
F_v/F_m	3	A3c	WT@27	SS@30a	4.61	<0.01
F_v/F_m	3	A3c	WT@27	SS@30b	5.82	<0.01
F_v/F_m	3	A3c	WT@27	SS@30c	5.28	<0.01
F_v/F_m	10	A3c	WT@27	SS@30a	7.18	<0.01
F_v/F_m	10	A3c	WT@27	SS@30b	7.70	<0.01
F_v/F_m	10	A3c	WT@27	SS@30c	6.47	<0.01
F_v/F_m	10	A3c	WT@30	WT@27	-6.36	<0.01
F_v/F_m	17	A3c	WT@27	SS@30a	4.88	<0.01
F_v/F_m	17	A3c	WT@27	SS@30b	6.60	<0.01
F_v/F_m	17	A3c	WT@27	SS@30c	4.67	<0.01
F_v/F_m	17	A3c	WT@30	WT@27	-6.68	<0.01
F_v/F_m	26	A3c	WT@27	SS@30a	8.08	<0.01
F_v/F_m	26	A3c	WT@27	SS@30b	8.22	<0.01
F_v/F_m	26	A3c	WT@27	SS@30c	8.69	<0.01
F_v/F_m	26	A3c	WT@30	WT@27	-5.59	<0.01
F_v/F_m	26	A3c	WT@30	WT@27	-11.21	<0.01
F_v/F_m	3 and 26	A3c	SS@30a	SS@30a	2.15	0.81

Appendix 4.2 Statistically significant pairwise comparisons (Tukey's post-hoc tests) in mean growth rates (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Durusdinium* (ITS2 type D1) populations during the 30°C reciprocal transplant experiment

			Pairwise comparison			
Trait	Day	ITS2 type	Population 1	Population 2	t-value	p-value
μ	17	D1	SS@30a	WT@27	-3.76	0.0243
μ	17	D1	SS@30a	WT@30	-3.89	0.0212
μ	17	D1	SS@30c	SS@30b	3.73	0.0253
$\Delta F/F_m'$	3	D1	WT@27	SS@30a	7.07	<0.01
$\Delta F/F_m'$	3	D1	WT@30	SS@30a	5.10	<0.01
$\Delta F/F_m'$	3	D1	SS@30c	SS@30b	3.86	0.04
$\Delta F/F_m'$	3	D1	WT@27	SS@30b	9.63	<0.01
$\Delta F/F_m'$	3	D1	WT@30	SS@30b	7.66	<0.01
$\Delta F/F_m'$	3	D1	WT@27	SS@30c	5.78	<0.01
$\Delta F/F_m'$	3	D1	WT@30	SS@30c	3.81	0.05
$\Delta F/F_m'$	10	D1	WT@27	SS@30a	7.18	<0.01
$\Delta F/F_m'$	10	D1	WT@30	SS@30a	4.16	0.02
$\Delta F/F_m'$	10	D1	WT@27	SS@30b	9.22	<0.01
$\Delta F/F_m'$	10	D1	WT@30	SS@30b	6.20	<0.01
$\Delta F/F_m'$	10	D1	WT@27	SS@30c	5.59	<0.01
$\Delta F/F_m'$	17	D1	WT@30	SS@30b	6.12	<0.01
$\Delta F/F_m'$	17	D1	WT@30	SS@30c	4.12	0.02
$\Delta F/F_m'$	26	D1	WT@27	SS@30b	6.10	<0.01
$\Delta F/F_m'$	26	D1	WT@30	SS@30b	4.61	<0.01
$\Delta F/F_m'$	26	D1	WT@27	SS@30c	4.00	0.03
$\Delta F/F_m'$	3 and 26	D1	SS@30a	SS@30a	5.35	<0.01
$\Delta F/F_m'$	3 and 26	D1	SS@30b	SS@30b	4.25	0.01
F_v/F_m	3	D1	WT@30	SS@30a	7.52	<0.01
F_v/F_m	3	D1	SS@30c	SS@30b	4.98	<0.01
F_v/F_m	3	D1	WT@27	SS@30b	5.77	<0.01
F_v/F_m	3	D1	WT@30	SS@30b	9.96	<0.01
F_v/F_m	3	D1	WT@30	SS@30c	4.98	<0.01
F_v/F_m	3	D1	WT@30	WT@27	4.19	0.02
F_v/F_m	10	D1	WT@27	SS@30a	5.30	<0.01
F_v/F_m	10	D1	WT@27	SS@30b	7.14	<0.01
F_v/F_m	10	D1	WT@30	SS@30b	4.87	<0.01
F_v/F_m	10	D1	WT@27	SS@30c	4.25	0.02
F_v/F_m	17	D1	WT@27	SS@30a	6.48	<0.01
F_v/F_m	17	D1	WT@30	SS@30a	7.59	<0.01
F_v/F_m	17	D1	WT@27	SS@30b	5.96	<0.01
F_v/F_m	17	D1	WT@30	SS@30b	7.07	<0.01
F_v/F_m	17	D1	WT@27	SS@30c	6.18	<0.01
F_v/F_m	17	D1	WT@30	SS@30c	7.29	<0.01
F_v/F_m	26	D1	SS@30b	SS@30a	-3.84	0.04
F_v/F_m	26	D1	WT@27	SS@30b	5.16	<0.01
F_v/F_m	26	D1	WT@30	SS@30b	4.59	<0.01
F_v/F_m	26	D1	WT@27	WT@27	5.70	<0.01
F_v/F_m	3 and 26	D1	SS@30a	SS@30a	7.71	<0.01
F_v/F_m	3 and 26	D1	SS@30b	SS@30b	6.31	<0.01

Appendix 4.3 Statistically significant pairwise comparisons (Tukey's post-hoc tests) in mean growth rates (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Fugacium* (ITS2 type F1) populations during the 30°C reciprocal transplant experiment

			Pairwise comparison			
Trait	Day	ITS2 type	Population 1	Population 2	t-value	p-value
μ	17	F1	SS@30b	WT@27	-3.81	0.02
μ	17	F1	SS@30b	WT@30	-3.60	0.03
μ	17	F1	SS@30b	SS@30a	-3.36	0.04
$\Delta F/F_m'$	17	F1	WT@27	SS@30a	-11.66	<0.01
$\Delta F/F_m'$	17	F1	WT@30	SS@30a	-12.01	<0.01
$\Delta F/F_m'$	17	F1	WT@27	SS@30b	-10.04	<0.01
$\Delta F/F_m'$	17	F1	WT@30	SS@30b	-10.40	<0.01
$\Delta F/F_m'$	17	F1	WT@27	SS@30c	-11.20	<0.01
$\Delta F/F_m'$	17	F1	WT@30	SS@30c	-11.56	<0.01
$\Delta F/F_m'$	26	F1	WT@27	SS@30a	-11.02	<0.01
$\Delta F/F_m'$	26	F1	WT@30	SS@30a	-15.55	<0.01
$\Delta F/F_m'$	26	F1	WT@27	SS@30b	-9.64	<0.01
$\Delta F/F_m'$	26	F1	WT@27	SS@30c	-10.16	<0.01
$\Delta F/F_m'$	26	F1	WT@30	SS@30c	-14.69	<0.01
$\Delta F/F_m'$	26	F1	WT@30	WT@27	-4.53	<0.01
$\Delta F/F_m'$	3 and 26	F1	SS@30a	SS@30a	5.93	<0.01
$\Delta F/F_m'$	3 and 26	F1	SS@30b	SS@30b	4.14	0.02
$\Delta F/F_m'$	3 and 26	F1	SS@30c	SS@30c	4.05	0.03
$\Delta F/F_m'$	3 and 26	F1	WT@27	WT@27	-6.96	<0.01
F_v/F_m	3	F1	SS@30c	SS@30a	3.89	0.04
F_v/F_m	3	F1	WT@27	SS@30a	4.76	<0.01
F_v/F_m	26	F1	WT@27	SS@30a	-6.90	<0.01
F_v/F_m	26	F1	WT@30	SS@30a	-10.21	<0.01
F_v/F_m	26	F1	WT@27	SS@30b	-4.81	<0.01
F_v/F_m	26	F1	WT@30	SS@30b	-8.12	<0.01
F_v/F_m	3 and 26	F1	SS@30b	SS@30b	4.53	<0.01

Appendix 4.4 Statistically significant pairwise comparisons (Tukey's post-hoc tests) for (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Fugacium* (ITS2 type F1) populations during the 31°C reciprocal transplant experiment

		Symbiodinium	Pairwise comparison			
Trait	Day	type	Population 1	Population 2	t-value	p-value
$\Delta F/F_m'$	3	F1	SS@31b	SS@31a	6.15	<0.01
$\Delta F/F_m'$	3	F1	SS@31c	SS@31a	5.78	<0.01
$\Delta F/F_m'$	3	F1	WT@31	SS@31a	5.60	<0.01
$\Delta F/F_m'$	10	F1	WT@31	SS@31a	-4.52	<0.01
$\Delta F/F_m'$	10	F1	WT@31	SS@31b	-4.26	0.01
$\Delta F/F_m'$	10	F1	WT@31	SS@31c	-4.54	<0.01
$\Delta F/F_m'$	10	F1	WT@31	WT@27	-4.69	<0.01
$\Delta F/F_m'$	17	F1	WT@27	SS@31a	-6.64	<0.01
$\Delta F/F_m'$	17	F1	WT@27	SS@31b	-5.90	<0.01
$\Delta F/F_m'$	17	F1	WT@27	SS@31c	-6.871	<0.01
$\Delta F/F_m'$	17	F1	WT@31	WT@27	6.24	<0.01
$\Delta F/F_m'$	26	F1	WT@27	SS@31a	-5.90	<0.01
$\Delta F/F_m'$	26	F1	WT@27	SS@31b	-5.62	<0.01
$\Delta F/F_m'$	26	F1	SS@31c	WT@27	-6.27	<0.01
$\Delta F/F_m'$	26	F1	WT@31	WT@27	5.50	<0.01
$\Delta F/F_m'$	3 and 26	F2	SS@31a	SS@31a	7.36	<0.01
F_v/F_m	17	F1	WT@27	SS@31a	-4.13	0.02
F_v/F_m	17	F1	WT@27	SS@31c	-4.35	0.0114
F_v/F_m	26	F1	WT@27	SS@31a	-7.47	<0.01
F_v/F_m	26	F1	WT@27	SS@31b	-5.08	<0.01
F_v/F_m	26	F1	WT@27	SS@31c	-4.66	<0.01
F_v/F_m	26	F1	WT@31	WT@27	5.98	<0.01
F_v/F_m	3 and 26	F1	SS@31a	SS@31a	6.92	<0.01
F_v/F_m	3 and 26	F1	SS@31b	SS@31b	4.17	0.02

Appendix 4.5 Statistically significant pairwise comparisons (Tukey's post-hoc tests) for, light-adapted effective quantum yields ($\Delta F/F'_m$) and dark-adapted maximum quantum yields (F_v/F_m) between *Fugacium* (ITS2 type F1) populations during the 33°C reciprocal transplant experiment

Trait	Day	*Symbiodinium* type	Pairwise comparison		t-value	p-value
			Population 1	Population 2		
$\Delta F/F'_m$	3	F1	SS@33b	SS@33a	-5.74	<0.01
$\Delta F/F'_m$	3	F1	SS@33c	SS@33b	5.14	<0.01
$\Delta F/F'_m$	3	F1	WT@33	SS@33b	5.33	<0.01
$\Delta F/F'_m$	3	F1	WT@27	SS@33b	8.33	<0.01
$\Delta F/F'_m$	10	F1	WT@27	SS@33a	4.07	0.02
$\Delta F/F'_m$	10	F1	WT@27	SS@33b	5.96	<0.01
$\Delta F/F'_m$	17	F1	WT@27	SS@33a	-5.39	<0.01
$\Delta F/F'_m$	17	F1	WT@27	SS@33b	-3.98	0.03
$\Delta F/F'_m$	17	F1	WT@27	SS@33c	-4.90	<0.01
$\Delta F/F'_m$	17	F1	WT@33	WT@27	5.50	<0.01
$\Delta F/F'_m$	3 and 26	F1	WT@27	WT@27	-6.6	<0.01
$\Delta F/F'_m$	3 and 26	F1	SS@33b	SS@33b	4.38	0.01
F_v/F_m	3	F1	SS@33b	SS@33a	-5.68	<0.01
F_v/F_m	3	F1	WT@27	SS@33b	7.64	<0.01
F_v/F_m	3	F1	WT@33	SS@33b	5.87	<0.01
F_v/F_m	3	F1	WT@27	SS@33c	4.06	0.026
F_v/F_m	10	F1	WT@27	SS@33a	9.57	<0.01
F_v/F_m	10	F1	WT@27	SS@33b	10.75	<0.01
F_v/F_m	10	F1	WT@27	SS@33c	10.16	<0.01
F_v/F_m	10	F1	WT@33	WT@27	-9.12	<0.01
F_v/F_m	26	F1	WT@33	WT@27	4.24	0.01
F_v/F_m	3 and 26	F1	SS@33b	SS@33b	6.77	<0.01
F_v/F_m	3 and 26	F1	WT@27	WT@27	-3.92	0.04

Appendix 4.6 Statistically significant pairwise comparisons (Tukey's post-hoc tests) for growth rate (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Fugacium* (ITS2 type F1) populations during the 34°C reciprocal transplant experiment

		Symbiodinium	Pairwise comparison			
Trait	Day	type	Population 1	Population 2	t-value	p-value
μ	17	F1	SS@34b	WT@34	3.814	0.02
$\Delta F/F_m'$	3	F1	WT@27	SS@34a	8.39	<0.01
$\Delta F/F_m'$	3	F1	WT@27	SS@34b	6.48	<0.01
$\Delta F/F_m'$	3	F1	WT@27	SS@34c	5.89	<0.01
$\Delta F/F_m'$	3	F1	WT@34	WT@27	-5.17	<0.01
$\Delta F/F_m'$	10	F1	WT@27	SS@34a	8.27	<0.01
$\Delta F/F_m'$	10	F1	WT@27	SS@34b	7.67	<0.01
$\Delta F/F_m'$	10	F1	WT@27	SS@34c	7.82	<0.01
$\Delta F/F_m'$	10	F1	WT@34	WT@27	-7.72	<0.01
$\Delta F/F_m'$	3 and 26	F1	WT@27	WT@27	-5.89	<0.01
F_v/F_m	3	F1	WT@34	SS@34a	5.26	<0.01
F_v/F_m	10	F1	WT@27	SS@34a	10.27	<0.01
F_v/F_m	10	F1	WT@27	SS@34b	9.04	<0.01
F_v/F_m	10	F1	WT@27	SS@34c	7.50	<0.01
F_v/F_m	10	F1	WT@34	WT@27	-7.71	<0.01
F_v/F_m	3 and 26	F1	WT@34	WT@34	-5.68	<0.01

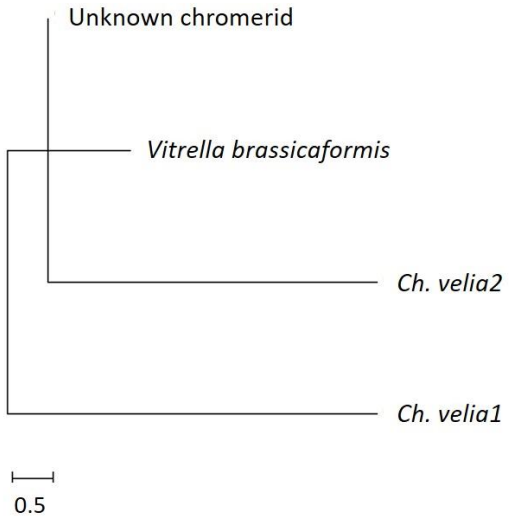
Appendix 4.7 Statistically significant pairwise comparisons (Tukey's post-hoc tests) for growth rate (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Gerakladium* (ITS2 type G1) populations during the 30°C reciprocal transplant experiment

			Pairwise comparison			
Trait	Day	*Symbiodinium* type	Population 1	Population 2	t-value	p-value
$\Delta F/F_m'$	3	G3	WT@30	WT@27	-4.08	0.024
$\Delta F/F_m'$	3 and 26	G3	SS@30b	SS@30b	5.81	<0.01
$\Delta F/F_m'$	3 and 26	G3	SS@30c	SS@30c	7.40	<0.01
$\Delta F/F_m'$	3 and 26	G3	WT@30	WT@30	7.19	<0.01
F_v/F_m	3	G3	WT@27	SS@30a	6.09	<0.01
F_v/F_m	3	G3	WT@27	SS@30c	5.98	<0.01
F_v/F_m	3	G3	WT@30	WT@27	-5.67	<0.01
F_v/F_m	3 and 26	G3	SS@30a	SS@30a	10.48	<0.01
F_v/F_m	3 and 26	G3	SS@30b	SS@30b	8.24	<0.01
F_v/F_m	3 and 26	G3	WT@30	WT@30	9.09	<0.01

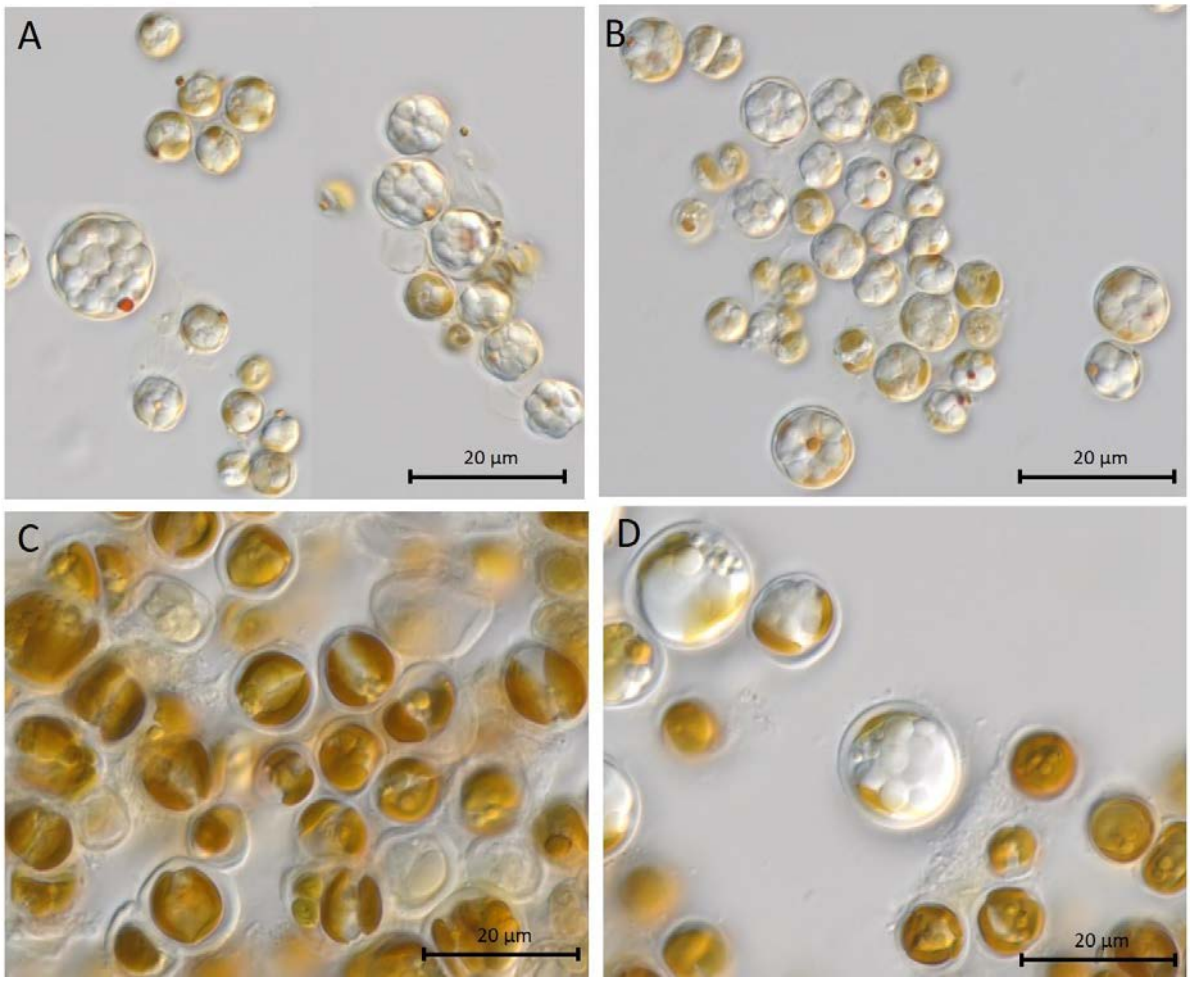
Appendix 4.8 Statistically significant pairwise comparisons (Tukey's post-hoc tests) for growth rate (μ), light-adapted effective quantum yields ($\Delta F/F_m'$) and dark-adapted maximum quantum yields (F_v/F_m) between *Gerakladium* (ITS2 type G1) populations during the 31°C reciprocal transplant experiment

		Symbiodinium	Pairwise comparison			
Trait	Day	type	Population 1	Population 2	t-value	p-value
μ	17	G3	WT@31	Wt@27	-4.228	0.01
μ	17	G3	SS@31a	WT@31	3.968	0.02
$\Delta F/F_m'$	3	G3	WT@31	SS@31a	-3.92	0.04
$\Delta F/F_m'$	3	G3	WT@31	SS@31b	-4.02	0.03
$\Delta F/F_m'$	3	G3	WT@31	WT@27	-4.09	0.02
$\Delta F/F_m'$	10	G3	WT@27	SS@31a	6.09	<0.01
$\Delta F/F_m'$	10	G3	WT@27	SS@31b	6.49	<0.01
$\Delta F/F_m'$	10	G3	WT@27	SS@31c	7.12	<0.01
$\Delta F/F_m'$	10	G3	WT@31	WT@27	-8.46	<0.01
$\Delta F/F_m'$	3 and 26	G3	SS@31c	SS@31c	5.42	<0.01
$\Delta F/F_m'$	3 and 26	G3	WT@31	WT@31	6.14	<0.01
F_v/F_m	3	G3	WT@31	SS@31a	-4.37	0.01
F_v/F_m	3	G3	WT@31	SS@31b	-4.06	0.02
F_v/F_m	3	G3	WT@27	SS@31c	5.24	<0.01
F_v/F_m	3	G3	WT@31	WT@27	6.44	<0.01
F_v/F_m	10	G3	WT@27	SS@31a	3.84	0.05
F_v/F_m	10	G3	WT@27	SS@31b	4.39	0.01
F_v/F_m	10	G3	WT@27	SS@31c	4.67	<0.01
F_v/F_m	10	G3	WT@31	WT@27	-6.00	<0.01
F_v/F_m	3 and 26	G3	SS@31a	SS@31a	7.29	<0.01
F_v/F_m	3 and 26	G3	SS@31b	SS@31b	6.44	<0.01
F_v/F_m	3 and 26	G3	SS@31c	SS@31c	7.55	<0.01
F_v/F_m	3 and 26	G3	WT@31	WT@31	9.69	<0.01

Appendix 5.1 Neighbour-joining distance tree (A) based on pairwise distances between the 18S sequences for the four chromerid microalgae. All bootstrap values (500 replicates) were <36. Phylogenetic evolutionary analyses were conducted using MEGA version 4 ((Tamura et al. 2007)



Appendix 5.2. Microscopy images using differential interference contrast of free-living cultures of *Vitrella brassicaformis* (A), the unknown chromerid (B), *C. velia1* (C) and *C. velia2* (D)



Appendix 5.3. Results from a BLASTn search of highly similar sequences using (database: nucleotide collection nr/nt). *these isolates are described as *Chromera velia* in Genbank and in their respective publications, however they were later described *as Vitrella brassicaformis* by Obornik et al. (2012) and originate from the same strain, first isolated by Moore et al. (2008) and also used in this study.

Accession number	Species name	Isolate	Clone	Score	Query cover (%)	Identity (%)	Expect value	Publication
JN986791.1	*Chromera velia*	Mdig4		3070	100	99	0	unpublished (2012)
DQ174731.1	**Vitrella brassicaformis*	CMS22		3066	100%	99	0	Moore et al. 2008
JN986790.1	*Chromera velia*	Mdig3		6053	100%	99	0	Cumbo et al. 2013
JN986789.1	*Chromera velia*	Mdig2		6048	100%	99	0	Cumbo et al. 2013
JN986792.1	*Chromera velia*	Mdig5		3042	100%	99	0	unpublished (2012)
JN935832.1	**Vitrella brassicaformis*	CvLp_vc08/1	JS494	3009	98%	99	0	Morin-Adeline et al. 2012
JN935830.1	**Vitrella brassicaformis*	CvLp_vc08/1	JS492	3009	98%	99	0	Morin-Adeline et al. 2012
JN935829.1	*Vitrella brassicaformis*	CvLp_vc08/1	JS491	3009	98%	99	0	Morin-Adeline et al. 2012
JN986788.1	*Chromera velia*	Mdig1		3005	100%	99	0	Cumbo et al. 2013
JN935834.1	**Vitrella brassicaformis*	CvLp_vc08/1	JS496	2882	98%	99	0	Morin-Adeline et al. 2012

Appendix 5.4. *In vitro* mean temperature, standard error and degrees of freedom (df) that ***Cladocopium goreaui*** **and the chromerids** experienced during the ***In vitro*** **temperature and diuron sensitivity** experiment for either 12 or 22 days.

Temperature exposure (days)	Temperature (°C)	Mean	Standard error (±)
12	27	26.82	0.004
12	30	30.01	0.003
12	31	31.12	0.012
12	32	31.91	0.011
22	27	26.76	0.004
22	30	30.01	0.002
22	31	31.10	0.007
22	32	31.91	0.006

Appendix 5.5. *In hospite* mean temperature, standard error and degrees of freedom (df) that *Acropora tenuis* and *Acropora millepora* larvae experienced during the two-week inoculation experiment.

Species	Temperature (°C)	Mean	Standard error (±)
A. tenuis	27	26.66	0.008
A. tenuis	30	30.06	0.002
A. tenuis	31	31.12	0.010
A. millepora	27	26.83	0.005
A. millepora	30	30.06	0.002
A. millepora	31	31.12	0.002

Appendix 5.6 Analysis of variance (ANOVA) output of linear mixed effects models for testing whether microalgal strain or temperature have a significant effect on photosynthetic traits; effective quantum yield ($\Delta F/F_m'$), maximum quantum yield (F_v/F_m), maximum pressure over photosystem II (Q_m) after 12 and 22 days of exposure to the different temperature conditions.

Trait	Days of exposure	Source	DF	F	p-value
$\Delta F/F_m'$	12	Strain	40	352.8	<0.0001
	12	Temperature	40	28.4	<0.0001
	12	Strain:temperature	40	39.7	<0.0001
F_v/F_m	12	Strain	40	294.5	<0.0001
	12	Temperature	40	18.5	<0.0001
	12	Strain:temperature	40	5.1	<0.0001
Q_m	12	Strain	40	31.7	<0.0001
	12	Temperature	40	4.6	0.0076
	12	Strain:temperature	40	10.4	<0.0001
$\Delta F/F_m'$	22	Strain	40	791.0	<0.0001
	22	Temperature	40	251.8	<0.0001
	22	Strain:temperature	40	110.4	<0.0001
F_v/F_m	22	Strain	40	806.1	<0.0001
	22	Temperature	40	520.7	<0.0001
	22	Strain:temperature	40	171.3	<0.0001
Q_m	22	Strain	40	36.6	<0.0001
	22	Temperature	40	3.6	0.0211
	22	Strain:temperature	40	4.6	<0.0001

Appendix 5.7. Analysis of variance (ANOVA) output of general linear models testing whether microalgal strain, diuron or their interaction has a significant effect on *Acropora tenuis* larval uptake or larval mortality after 14 days of exposure to the different microalgal strains, diuron and temperature conditions.

Trait	Temperature (°C)	Source	DF	Chisq	p-value
Larval uptake	27	Strain	4	49819	<0.0001
		Diuron	1	8074	<0.0001
		Strain:diuron	4	25	<0.0001
	30	Strain	4	1352	<0.0001
		Diuron	1	284	<0.0001
		Strain:diuron	4	427	<0.0001
	31	Strain	4	62	<0.0001
		Diuron	1	2.1	0.145
Larval mortality	27	Strain	4	41	<0.0001
		Diuron	1	4.0	0.047
	30	Strain	4	8.8	0.066
		Diuron	1	4.2	0.040
	31	Strain	4	44	<0.0001
		Diuron	1	6.1	0.013
		Strain:diuron	4	25	<0.0001

Appendix 5.8. Analysis of variance (ANOVA) output of general linear models testing whether microalgal strain, diuron or their interaction has a significant effect on *Acropora millepora* larval uptake or larval mortality after 14 days of exposure to the different microalgal strains, diuron and temperature conditions.

Trait	Temperature (°C)	Source	DF	Chisq	p-value
Larval uptake	27	Strain	4	32594	<0.0001
		Diuron	1	5148	<0.0001
		Strain:diuron	4	90	<0.0001
	30	Strain	4	435	<0.0001
		Diuron	1	3.6	0.056
		Strain:diuron	4	27	<0.0001
	31	Strain	4	59	<0.0001
		Diuron	1	26	<0.0001
		Strain:diuron	4	10	0.039
Larval mortality	27	Strain	4	27	<0.0001
		Diuron	1	23	<0.0001
	30	Strain	4	5.2	0.265
		Diuron	1	5.5	0.019
		Strain:diuron	4	9.5	0.050
	31	Strain	4	7.0	0.135
		Diuron	1	0.74	0.390
		Strain:diuron	4	9.8	0.044