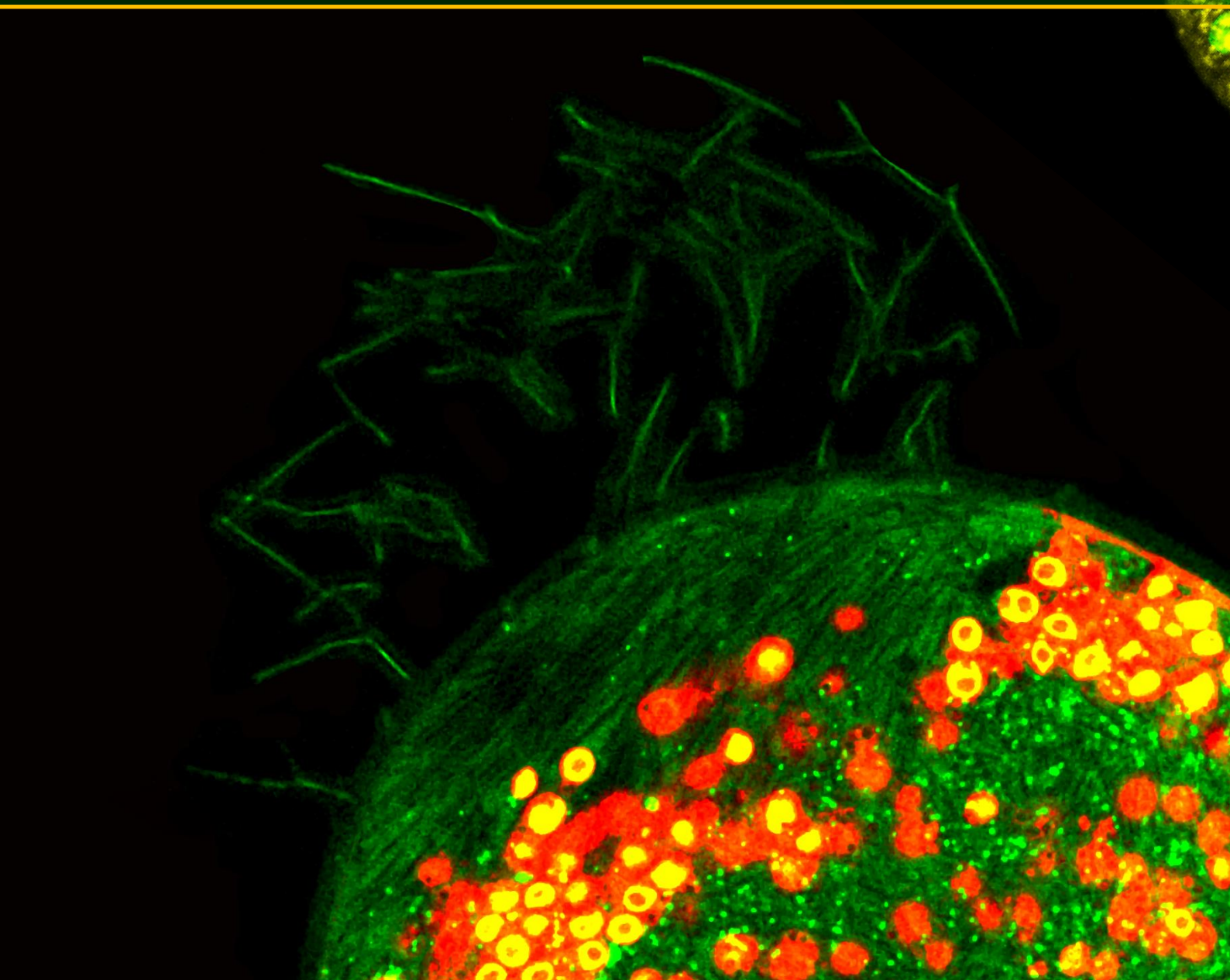
It's Getting Hot in Here: Ecological and Evolutionary Responses of Photo-Symbiotic Mutualisms to Warming

Benjamin Andrew Makin



Submitted by

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To the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

September, 2019

I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Signature: 

“All science is either physics or stamp collecting”

- Ernest Rutherford*

PREFACE

Four years ago – before I set sail for Cornwall in order to embark upon a PhD – I met Prof. Richard Kenchington, who acted as director for the Great Barrier Reef Marine Park Authority between 1977 and 1999. While Prof Kenchington clearly cared deeply for coral reefs, and the biodiversity that they support, he was particularly delighted to hear that I would spend the next few years studying photo-symbiosis – not in corals – but in ciliates. Corals and the bleaching phenomenon (and the devastating photographs of bleached reefs that they routinely produce in the news) receive an abundance of research attention, but is it always justified? Of course, reefs are highly important for ecosystems, supporting around one-third of all species of marine fish and contributing a great deal to rates of primary production. However, researchers are becoming increasingly aware that photo-symbiosis is widespread in aquatic environments; many of these photo-symbioses are microbial, with hosts that are invisible to the naked eye. Is ‘bleaching’ and its consequences any less severe in these microbes? What will be the likely impacts of global warming on these organisms? These are questions that, by comparison with corals, are neglected. This is despite the clear benefits of using microbes in a laboratory environment: with comparable ease, one can grow large populations of microbial photo-symbioses over many generations in order to investigate evolutionary responses to warming

(something which is difficult in corals). It is partly for these reasons, and primarily because I wish to obtain a science PhD and not a stamp-collecting one, that I am now going to extend Rutherford's definition* of 'physics' to include the study of metabolism (this should not be a problem, since metabolism is fundamentally thermodynamics applied to living organisms). In this thesis, I will detail studies that investigate the responses of photo-symbioses to warming: my investigations will span the time scales of seconds (i.e. metabolism) to days (i.e. ecological responses) to months (i.e. evolutionary responses).

For Rod

The greatest singer ever

Acknowledgements

I wish to thank my supervisor, Dr Chris Lowe, who made it all possible. We shared a fair few cheeky coffees, the odd civilised beer, and many great chats. Thanks, Chris. Thanks also to my top lap buddy, Dr Sam Barton; we enjoyed perhaps too many late-night oxylab sessions, but at least we had Rod Stewart on the playlist. Because it was all for love, baby. Thanks also to my top Masters lab buddy, fellow radio connoisseur and Dynamite Valley Brewery enthusiast, Sam Oyesiku-Blakemore. We well and truly rocked the Cornish airways, brother. I guess I should thank Steph, too, or else she'll be disappointed (not that she, or indeed anyone, will ever read this). I also offer a big 'thank you' to my fellow office buds, Ben Toulson (who plays a MEAN guitar), Jared Wilson-Aggarwal, Govinder Pawar and Beth Roberts.

ABSTRACT

In response to contemporary climate change, ecologists now possess a great deal of knowledge about the specific, short-term impacts of warming on globally important symbiotic mutualisms. This is particularly true in the case of the coral-zooxanthellae association, where physiological stress can drive the loss of symbionts from hosts (“bleaching”) and can thus lead to the breakdown of the association. In terms of future predictions, this potentially risks the provision of the important ecosystem services that they currently provide in a future, warmer world. However, there are three blatant limitations with this perspective and in the wider field of symbiosis break down. Firstly, our understanding of when symbioses break down in response to the environment is highly case-specific and invokes specific mechanisms such as host regulation of symbionts. General, underlying principles that govern the sensitivity of photo-symbioses to temperature would usefully be elucidated and have been called out for in the literature. Second, the coral symbiosis is representative of a broader group of symbioses in which photosynthetic algae reside within heterotrophic hosts (i.e. “photo-symbioses”). Many such associations involve unicellular algae residing within unicellular hosts; these are comparatively poorly studied, but are now recognized to be highly abundant and diverse in aquatic ecosystems, underpin a great deal of primary production, and play a key role in aquatic food-webs and rates of heterotrophic grazing. Thus, by comparison, the responses of these microbial photo-symbioses have been neglected. Third, the longer term (evolutionary) responses of photo-symbioses are inherently difficult to study directly, thus much of our understanding of their potential evolutionary trajectories in response to warming is derived from theory, modelling, comparative phylogenetics and extrapolation from

short-term ecological responses. Recent reviews of such studies have called for support from direct empirical studies. In this thesis, we address these three limitations through the use of the tractable, microbial photo-symbiosis – the *Paramecium bursaria-Chlorella spp*. association. We address the specific research questions: Firstly, can we understand the responses of photo-symbiosis based on simple metabolism and growth dynamics? Second, how will microbial photo-symbioses respond to warming – and what could the wider consequences for the ecosystems be – over long (evolutionary) time scales? In chapter 2, we found that the different nitrogen sources used by free-living and symbiotic algae caused differences in metabolic thermal response, suggesting that symbiont metabolism reacts differently to free-living autotrophs. In chapter 3, we found that symbiont abundance within hosts closely followed symbiont growth rate with the opposite pattern in bacterivory, suggesting that departures from the thermal growth optimum for symbionts explained the 'bleaching' of, and increased heterotrophy in, the photo-symbiosis. In chapter 4, we found that ~10.5 months (~21 generations) under prolonged warming caused an increase in the thermal optima for holobiont growth while symbionts isolated from the long-term warming treatment were able to grow on inorganic nitrogen sources, suggesting that they had gained/re-gained the capacity for free-living growth. This suggests that warming produced an adaptive growth response in holobionts but also appeared to drive the evolution of increased autonomy in symbionts, representative of two divergent evolutionary trajectories. In the context of the ecological role of photo-symbioses, we also found that warm-adapted holobionts had significantly lower rates of primary production (strikingly, net primary production rates approached ~0), suggesting that the ecological function of photo-symbioses can change substantially with long-term warming. Taken together,

the research in this thesis suggests that symbiont physiology underpins the responses of photo-symbiosis to warming, in answer to our first research question. Second, we present evidence for divergent trajectories followed by photo-symbioses with long-term warming; thus, further work that investigates whether these outcomes would be possible in nature are now of paramount importance and will help predict the likely fate of photo-symbiosis in a warmer world.

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Chapter 1: Introduction

Background

It is well known that symbioses – in their broadest sense, as close associations between separate species – carry an ubiquitous importance throughout biology. They have enjoyed a striking involvement in key evolutionary events; an endosymbiotic interaction transplanted the energy-generating potential of proton gradients, enclosed within membranes, into the symbiotic ancestors of eukaryotes (e.g. Lane, 2014; Frank, 1995), where it arguably paved the way for all complex multicellular life (Lane, 2014). Earlier still, the first genomes, sometime near the origin of life, probably formed via symbiotic associations between separate replicators (Frank, 1995). A symbiosis between fungi and the roots of land plants played an essential role in allowing plants to invade the non-aquatic Earth (Kiers *et al*, 2010); the list of examples is extensive. In the same vein, symbioses play an equally vital role in extant ecology. Remarkably, every species on Earth depends upon mutualistic (i.e. where both partners benefit) symbioses (Kiers *et al*, 2010). Some well-known mutualistic symbioses include plant-pollinator relationships (upon which over half of all extant flowering plants depend) (Landry, 2010), human gut microbiota (Li *et al*, 2008), numerous photosymbioses providing over 50% of the Ocean's primary productivity (Bailly *et al*, 2014) (e.g. Cnidarian-Dinoflagellate symbioses such as the Reef-accreting coral-zooxanthellae partnership) (Davy *et al*, 2012; Weis, 2010) and much of the planet's freshwater biomass (Sonntag *et al*, 2011; Summerer *et al*, 2008) (e.g. ciliate-algae symbioses such as the common textbook example *Paramecium bursaria-Chlorella* spp.) (Nowack & Melkonian, 2010)

and the associations between chemosynthetic microorganisms and certain marine organisms (enabling non-photosynthetic primary production in deep-sea ecosystems, such as those found at hydrothermal vents (e.g. Smith 2012)).

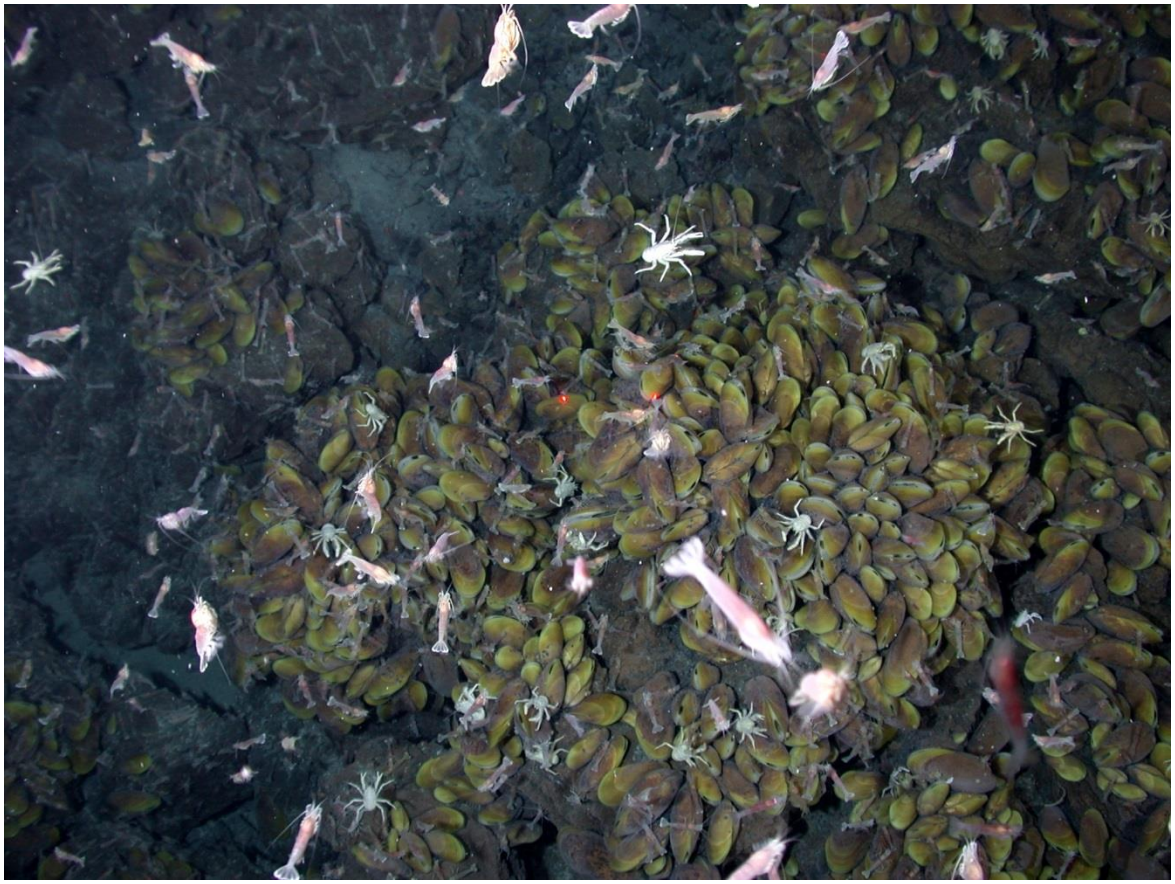


Image: fauna at a hydrothermal vent. Image courtesy of Submarine Ring of Fire 2006 Exploration, NOAA Vents Program.

Whilst their importance is unequivocal, there has classically been much debate over the rather more mundane semantics associated with the term *symbiosis* (e.g. West *et al*, 2007; Bronstein, 1994; Law & Dieckmann, 1998, Boucher *et al*, 1982). Traditionally, symbiotic relationships have been termed *parasitic* (whereby one of the interacting species suffers a cost associated with infection), *commensalistic* (whereby the association is cost-neutral) or *mutualistic* (whereby both species benefit from symbiosis) (see Boucher *et al*, 1982). Certain symbioses have been

notoriously difficult to assign to one of these categories, occupying one or the other depending on the context, or existing somewhere 'in-between' (Dimijian, 2000). Since this now outdated perspective, a growing body of literature has produced a revised view of symbiosis (see Bronstein, 1994), which shifts the nature of these arbitrary labels towards outcomes; all symbioses exist on a scale from parasitism to mutualism, and the biotic and abiotic conditions (i.e. the context) that the symbiosis exists within, in any given location in time or space, affects the dynamic outcome (Bronstein, 1994). In this way, a mutualism can become a parasitism and vice versa (e.g. Shapiro *et al*, 2016), possessing a property analogous to the plasticity of a phenotypic trait – which, too, is left at the mercy of context.

Regrettably, this deeper understanding of symbiosis derives largely from the study of the impacts of climate change and contemporary environmental degradation on symbiotic relationships (reviewed in Kiers *et al*, 2010; see Weis, 2010; Hoffmann & Sgrò, 2011). Unsurprisingly, rapidly changing environments carry the potential for significant shifts in the outcomes of symbioses; of particular concern are symbioses that are currently mutualistic (Kiers *et al*, 2010) (i.e. symbioses carrying the traditional definition *mutualisms,* but which could be specifically defined as symbioses displaying mutualistic outcomes under current conditions (Bronstein, 1994)), upon which much of the planet's biodiversity hinges (e.g. Bailly *et al*, 2014; Sonntag *et al*, 2011; Summerer *et al*, 2008). In what ways could such symbioses degrade in response to climate change? Theoretically, one way that mutualisms may respond is by becoming antagonistic; that is, they can become parasitic (Sachs & Simms, 2006). However, phylogenetic evidence suggests that shifts to parasitism are relatively rare (Sachs & Simms, 2006; Sachs, Skophammer & Regus, 2011) and

may often be restricted owing to irreversible losses of genes (Moran & Wernegreen, 2000). Furthermore, work on fungal-algal mutualisms suggests that mutualisms only break down when successful alternatives (such as symbiont switching or strategies to extract nutrients from the environment) evolve (Werner et al. 2018). However, partners may instead revert to an autonomous life history, and phylogenetic evidence suggests that this could be a common endpoint to mutualism by comparison (Sachs & Simms, 2006). As a result of the phylogenetic evidence, in a particularly charismatic paper, Frederickson, 2017 rebukes the notion that mutualisms are commonly sensitive to breakdown over evolutionary timescales.

Despite this, numerous studies have observed the breakdown of mutualisms in response to environmental stressors such as warming over shorter time periods, raising concerns for the future trajectories of mutualisms. Studies that demonstrate ecological, short-term breakdown now cover a wide range of symbioses, many of which are of notable importance for their wider ecosystems (Werner et al. 2018; Kiers et al. 2010) and include those between Cnidarians and Dinoflagellates (i.e. the well-documented ‘coral bleaching’, e.g. Lesser, 2011), plants and pollinators, plants and their associated rhizospheres, plants and dispersers and certain plants and their resident ant colonies (Kiers et al. 2010). For example, recent research has shown that environmental stress could cause the extinction of fig pollinators (Harrison, 2000; Jevanandam, Goh & Corlett, 2013), bacterial symbionts of stink bugs, beetles and other insects can be lost as a result of warming (Wernegreen, 2012; Kashkouli, Fathipour & Mehrabadi, 2019; Kikuchi et al 2016; Prado et al. 2010; Six & Bentz, 2007; Dysthe, Bracewell & Six, 2015), defensive symbioses can break down in response to warming, for example in aphid-bacteria and ant-plant mutualisms

(Doremus et al. 2018; Mooney et al. 2019; Fitzpatrick et al. 2014), the pathogenicity of *Wolbachia* symbioses can be increased with warming (Rohrscheib et al. 2016), nitrogen addition (e.g. through fertilisers) favours less cooperative mutualists in the legume–rhizobium mutualism (Weese et al. 2015), the timing of pollinator mutualisms can be disrupted due to changes in climate (Robbirt et al. 2014; Warren & Bradford, 2014; Warren, Bahn & Bradford, 2011), extreme heat events can break down temperate pollinators (Sutton et al. 2018) and tree-scatterhoarder mutualisms can transition towards antagonism in response to environmental stress (Sawaya et al. 2018). Studies have also observed the transitioning of mutualisms 'in the other direction' (i.e. from parasitism to mutualism). For example, recent research has shown that *Wolbachia* can evolve to become mutualistic in natural populations of *Drosophila* (Weeks et al 2007; note that this study followed *Drosophila* populations for 20 years), mutualism can evolve in experimental pathogenic virus populations (Shapiro and Turner, 2018), a fungal pathogen can evolve into a mammalian gut symbiont (Tso et al. 2018), a defensive symbiosis can rapidly evolve from a parasitic interaction (King et al. 2016), a plant pathogen can evolve into a legume symbiont (Marchetti et al. 2010; Su Hua Guan et al. 2013) and plant-virus parasitisms can become mutualistic (Hily et al. 2016). Research has also shown that algal-bacterial interactions can transition from antagonism to commensalism (Gonzalez-Olalla et al. 2018) and – perhaps most strikingly – a range of studies have documented the establishment of novel mutualisms from previously non-interacting partners. For example, novel symbioses have been established between yeast and algae (Naidoo et al. 2019), bacteria and archaea (Hillesland & Stahl, 2010), fungi and algae (Hom & Murray, 2014), bacteria and insects (Hosokawa et al. 2016), different bacterial species (Hosoda et al. 2011), bioluminescent bacteria and squid (Schuster, Perry &

Cooper, 2010) and ciliates and cyanobacteria (Ohkawa et al. 2011). At the very least, I argue that this wide range of studies serves as a 'proof of concept' for the dynamic nature of symbioses, a view that other researchers have taken (e.g. Kiers et al. 2010). Despite phylogenetic evidence suggesting that transitions between mutualism and parasitism have occurred relatively rarely over macroevolutionary timescales (Sachs & Simms, 2006; Sachs, Skophammer & Regus, 2011; Moran & Wernegreen, 2000; Frederickson, 2017), this 'proof of concept' is arguably reason for concern for the trajectories of mutualisms and their long-term management, such as in corals (Baker et al. 2008), in the face of climate change.

The contribution of this thesis

Throughout the research presented in this thesis, we employ tractable, microbial photosymbiotic organisms under laboratory conditions – the ciliated protist *Paramecium bursaria* and its algal symbionts, *Chlorella* spp (e.g. see Lowe, 2016; Karakashian, 1975; Kodama et al, 2014; Box 1) – in order to investigate the ecological resilience and evolutionary capacity of photo-symbiotic partnerships in response to experimental warming. There are three key justifications for this work. 1) The archetypal example of warming-induced photo-symbiosis breakdown is the coral-zooxanthellae symbiosis that underpins coral reef ecosystems, where temperature increase can lead to the stress-associated expulsion of symbionts from their hosts (e.g. Weis, 2008). However, coral-symbiont associations represent just one example of widespread and important photo-symbioses. Many other photo-symbioses involve unicellular algae residing within unicellular hosts; these are comparatively poorly studied, but are now recognized to be highly abundant and

diverse in aquatic ecosystems (Sonntag et al. 2011; Summerer et al. 2008; Sanders, 1991, 2011; Decelle, Colin & Foster, 2015), underpin a great deal of primary production (Baldauf, 2008) and, when considered alongside photo-mixotrophs, can often dominate ecosystem bacterivory (Berninger et al. 1992; Unrein et al. 2007; Zubkov & Tarran, 2008; Hartmann et al. 2012). Consequently, understanding the responses of unicellular photosymbioses to warming is likely to be important in understanding the broader ecological impacts of environmental warming for aquatic ecosystems. 2) Much of the current perspective on mutualism degradation is derived from theory and phylogenetics (e.g. Sachs & Simms, 2006; Sachs, Skophammer & Regus, 2011; Moran & Wernegreen, 2000; Werner et al. 2018; Frederickson, 2017), and extrapolation from short-term ecological responses (i.e. studies held over short time periods; e.g. Kiers et al. 2010; Lesser, 2011; Harrison, 2000; Jevanandam, Goh & Corlett, 2013; Wernegreen, 2012; Kashkouli, Fathipour & Mehrabadi, 2019; Kikuchi et al 2016; Prado et al. 2010; Six & Bentz, 2007; Dysthe, Bracewell & Six, 2015; Doremus et al. 2018; Mooney et al. 2019; Fitzpatrick et al. 2014; Robbirt et al. 2014; Warren & Bradford, 2014; Warren, Bahn & Bradford, 2011; Sutton et al. 2018; Sawaya et al. 2018; Rohrscheib et al. 2016), and questions about whether mutualisms will be subject to change over evolutionary time in the face of global warming are still of paramount importance (reviewed in Kiers et al. 2010). Indeed, in an informal critique by Kiers et al. 2010, the authors found only 15 out of 179 studies on mutualism disruption that included an empirical evolutionary component. 3) There is a great need to identify overarching principles and mechanisms in relation to symbiotic outcomes in extant mutualisms, especially with regard to photosymbiosis (Dupont & Pörtner, 2013; Bailly *et al*, 2014). For example, metabolic rates, which set abiotic constraints upon the 'pace of life' (Brown *et al*, 2004), are potentially powerful

general variables underlying symbiotic outcome, since a fundamental way in which conflicts between symbiotic organisms may arise is via differences in their metabolic responses (e.g. mismatched thermal optima) (Wernegreen, 2012). Recent work has demonstrated success in using elements of the metabolic theory of ecology (MTE) to explain the thermal niche of a broad range of phytoplankton (Barton et al. 2018; 2020) and the thermal adaptation of phytoplankton in the face of warming (Padfield et al. 2016; Schaum et al. 2017), yet these ideas are yet to be applied to photo-symbiosis (see Box 2).

Box 1: The *Paramecium bursaria: Chlorella spp*. photo-symbiosis

Symbionts of protozoa include prokaryotes and eukaryotes, and include a wide array of parasites as well as mutualists (Taylor & Sanders, 2010); the fitness effects of some symbionts may vary based on context or exist somewhere 'in between' (i.e. they can be commensals; Dimijian, 2000). Arguably the most striking are the killer particles of ciliates: these symbiotic bacteria transform hosts into 'zombie' conspecific killers that cause the death of sensitives by proximity, providing competitive advantages to infected hosts (Schrallhammer, Martina & Martina 2010). Interestingly, some protists may skirt the responsibilities of hosting symbionts while still gaining some of the benefits of the would-be symbiosis. For example, some marine ciliates consume algae and retain their chloroplasts (McManus et al. 2018); in this way, they become functionally mixotrophic in what is known as kleptoplastidy (the reader may be familiar with the particularly charismatic example found in the sea slug, *Costasiella kuroshimae*, informally referred to as 'leaf sheep'; see Christa et al.

2014). Perhaps the most obvious eukaryote symbionts of protozoa, however, are *Chlorella* algae (Taylor & Sanders, 2010); these symbionts are best studied in the ciliate, *Paramecium bursaria,* where they form a well-known photo-symbiosis (Karakashian, 1975). They also form associations with flagellates, amoebas and metazoans, such as corals, anemones and *Hydra* (e.g. Davy, Allemand & Weis, 2012; Douglas, 1994).

Image: *Costasiella Kuroshimae*. Author: Alif Abdul Rahman. This file is licensed under the Creative Commons Attribution-Share Alike 2.0 Generic license; Wikimedia Commons. No changes were made.

In some cases, benefits can be elucidated for hosts and symbionts, and thus the relationship between the algae and the host is mutualistic. Benefits for hosts can include the supply of photosynthates donated by symbionts; this is sometimes characterized by positive phototaxis (Taylor & Sanders, 2010), in which symbiotic hosts migrate in the direction of the sources of light stimuli. In *P. bursaria*, this can be demonstrated in the lab; dense accumulations of cells can be observed when populations are exposed to high PAR (Summerer et al. 2009). Indeed, symbionts release ~57% of fixed carbon to the host (Johnson, 2011), primarily as maltose (Ziesenisz, Reisser and Wiessner 1981). Other benefits in the *P. bursaria* photo-

symbiosis for hosts include photo-protection: symbionts can form intracellular 'umbrella' structures that protect the host cell from UV radiation (Summerer et al. 2009). In return, the symbiotic algae receive nitrogen from hosts; although the precise compounds remain to be elucidated, one of the best candidates is glutamine (He et al. 2019). Another key benefit of the association for symbionts in natural environments is protection from viral threats (Kodama & Fujishima, 2009 and references therein); in native freshwater, the titre of a *Paramecium bursaria Chlorella* virus (PBCV-1) can reach 100000 plaque-forming units per millilitre (Grimsley et al. 2012).

However, there is debate over whether the *P. bursaria-Chlorella spp*. photo-symbiosis is truly mutualistic, or whether it could better be understood as a host which exploits its symbionts (Lowe et al. 2016; Sørensen et al. 2019; Minter et al. 2018; Dean et al. 2016). This view is gaining momentum partly because many of the elucidated regulation mechanisms in the symbiosis are host-derived (see Sørensen et al. 2019). For example, the host is known to 'choose' its symbionts. This occurs during a recognition step, by which algae exposed to *P. bursaria* hosts are enclosed within a 'zipper-like' structure if they express the required cell wall carbohydrate structures (Reisser, 1992). This process has now been resolved at the ultrastructural level (Kodama & Fujishima, 2009): algae are first enveloped into digestive vacuoles. Budding of the digestive vacuole membrane occurs, producing small vacuoles containing each single 'accepted' alga; these then develop into perialgal vacuoles (PVs) which continue to resist degradation provided a number of requirements are met (Kodama & Fujishima, 2009). These requirements (and the degradation of the PV membrane that occurs should they not be met) could be interpreted as host

sanctions – mechanisms by which hosts 'punish' non-cooperators (see Foster et al. 2017; Sachs et al. 2004; Edwards, 2009). For example, photosynthetic production is required to maintain the structure of the PV (Kodama & Fujishima, 2008); the proper location within the host cell (i.e. the position of the trichocysts – 'harpoon-like' structures that can be used in host defence against antagonists) must be maintained (Kodama & Fujishima, 2009) and a light-induced factor must be released by symbionts (Kodama & Fujishima, 2014). Together, these mechanisms likely function

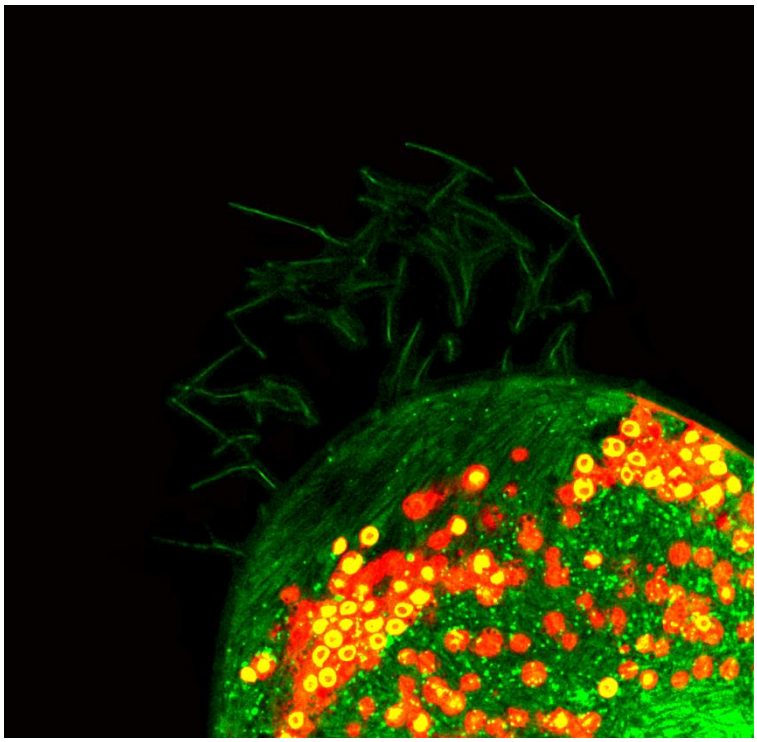


Image: *Paramecium bursaria* showing discharged trichocysts.

to maintain cooperative symbionts while removing those that resist control, are not productive, or that have become less productive due to environmental factors (e.g. changes in light intensity). The host is also capable of restricting its nitrogen provisioning to symbionts, and it may use this as a method to control the abundance of symbionts (i.e. symbiont load) in response to abiotic perturbation and associated changes in the relative 'value' of the symbionts (Lowe et al. 2016). In addition,

potential mechanisms by which hosts may 'reward' cooperative symbionts have been identified: for example, host-derived Ca^{2+} inhibits serine uptake into symbionts while glucose increases the uptake (Kato and Imamura 2008a; 2008b). If symbiont-derived maltose is broken down to glucose by hosts, this could represent a reward system for cooperative symbionts (Sørensen et al. 2019). The partnership shows signs of strong physiological interdependence: circadian rhythm of hosts and symbionts are linked (Miwa, 2009) while the cell division of symbionts is regulated and linked to host cytoplasmic streaming, enabling vertical transmission of symbionts (Takahashi et al. 2007). However, the host can often retain the ability to discard its symbionts and live symbiont-free (e.g. e.g. Tonooka & Watanabe, 2002) and/or acquire new populations from their surroundings via uptake into vacuoles (Kodama & Fujishima, 2009), while symbionts can sometimes retain the ability to grow autonomously (note that the capacity for autonomy in hosts and symbionts varies between different strains; Minter et al. 2018).

Regardless of whether the association is primarily mutualistic or exploitative, the term 'mutualism' is often used from a host perspective and may encompass relationships that involve exploitation of the symbionts (Hoang, Morran & Gerardo, 2016). This may be true in corals (Wooldridge, 2010) as well as in *P. bursaria* (Lowe et al. 2016). Note that a key reason for any conclusions drawn is likely to be the biotic and abiotic context that studies have invoked: for instance, in a laboratory study by Lowe et al. 2016, the lack of PBCV-1 may reduce the benefit of the symbiosis for symbionts, thus skewing the cost-benefit balance as a result of the simplified experimental system that the study necessarily examines. Thus, any discussion about the cost-benefit balance of the symbiosis requires assessment of

the biotic and abiotic conditions under scrutiny. For example, Kiers and West, 2016 likened the escape of symbionts from hosts at low light observed in the Lowe et al. 2016 study to the infamous Alcatraz prison: symbionts – the prisoners in this scenario – may be evolutionarily 'dissuaded from escape' due to poor conditions outside the symbiosis. This could be especially true if PBCV-1 is present in the environment, perhaps akin to armed guards if we are to build upon the Alcatraz metaphor.

Phylogenetically, *P. bursaria* is the earliest differentiated species among the five *Paramecium* species (He et al. 2019). However, the *P. bursaria-Chlorella spp*. photo-symbiosis has had a turbulent evolutionary history; the *Chlorella* group is polyphyletic and shows signs of repeated loss and gain of symbiosis (Hoshina & Imamura, 2009). *P. bursaria* likely first inherited two key strains (termed the 'European' and 'American' strains), following which one host population "chose" one of the strains and branched away from the other (Hoshina & Imamura, 2009). After this 'symbiont selection' event, the repeated loss, re-acquisition and switching of symbiont strains has apparently occurred. Particular genes that have been important in the establishment of symbiosis in *P. bursaria* have now been identified, utilising phylogenetic evidence and a series of ingenious gene knockout experiments that delivered siRNA to hosts via plasmids contained in bacterial prey (He et al. 2019). In addition, genes involved in the nitrate assimilation pathway have shown to be degraded independently at least twice in the *P. bursaria-Chlorella spp.* system and also in *Hydra viridissima* (Kamako et al. 2005; Hamada et al. 2018; Kato & Imamura, 2009). This could have occurred as a result of co-adaptation in symbiosis for metabolic efficiency reasons (Hamada et al. 2018), as a flux balance analysis

model has previously predicted (Sørensen et al. 2016), though this remains to be tested (see Chapter 2).

Box 2: Could metabolism prove insightful in understanding the thermal responses of photo-symbiosis?

The metabolic theory of ecology (MTE) – which posits that the rates of metabolism are fundamental in shaping a wide suite of patterns in ecology (Brown et al. 2004) – could prove insightful in the study of photo-symbiosis. Photo-symbiosis is typically viewed as a mutualism (although see Box 1), where a key benefit to both partners is delivered by trade in metabolites (e.g. Johnson, 2011). Since temperature underpins metabolic rate, and thus the rate at which metabolites are produced (and made available for trade), a key way in which the cost:benefit balance of photo-symbiosis could potentially be shifted is via metabolic thermal responses.

Due to the higher thermal sensitivity of respiration compared to photosynthesis (e.g. López-Urrutia et al. 2006; Anderson-Teixeira et al. 2011; Yvon-Durocher et al. 2010, 2012), warming tends to reduce the carbon fraction made available by

photosynthesis after accounting for respiratory losses; this balance is referred to as ‘carbon-use efficiency (CUE)’ (Padfield et al. 2016; see Figure 1). However, this response is potentially strongly modified by symbiosis. For example, there are likely to be strikingly different energetic costs associated with the use of different nitrogen sources (Sanz-Luque et al. 2015). These metabolic costs will be highly dependent on temperature; a fundamental determinant of metabolic rate (Brown et al. 2004). Consequently, it is reasonable to expect that the thermal responses of free-living algae and symbiotic algae (that have become dependent on host-derived nitrogen sources such as glutamine; Albers et al. 1982; He et al. 2019) could differ.

If CUE declines with warming, it is likely to increase the cost of photosynthate transfer for symbionts and decrease the benefit of harbouring symbionts for hosts (because symbionts will likely have less photosynthate available for trade, driving up its ‘price’ (Sørensen et al. 2016)). What responses might we expect as a result of this constraint? Firstly, CUE could result in constrained algal growth (Barton et al. 2018), potentially degrading the autotrophic capacity of hosts through the loss in algal biomass (i.e. “symbiont load”; the abundance of symbionts within hosts). In addition, hosts could actively evict ‘costly’ symbionts in response to declining CUE and/or symbionts could abandon the mutualism because the cost of metabolite trade is too high; all of these outcomes could result in the breakdown of symbiosis. Alternatively (and perhaps more optimistically), compensation mechanisms could emerge at high temperature. For example, hosts could increase their symbiont loads (i.e. they could exert control over the abundance of symbionts contained within them; Lowe et al, 2016) to make up for the ‘par capita’ reduction in the relative volume of photosynthate provision, or hosts and symbionts could evolve metabolic traits – as

have been observed in free-living phytoplankton (Padfield et al. 2016; Schaum et al. 2017) – that restore CUE.

In addition, warming has been shown to drive up the rates of heterotrophic process such as bacterivory more rapidly than autotrophic processes. Previous work on a different form of mixotroph (i.e. chloroplast-bearing protists capable of heterotrophic grazing) demonstrated increased grazing rates with warming (Wilken et al. 2013), and the authors reconciled their findings with general observations that the rates of heterotrophic processes tend to increase more rapidly with temperature than photosynthetic autotrophy (e.g. Rose & Caron, 2007; and see Wilken et al. 2018 and references therein).

In conclusion, changes in the metabolic rates in photosymbiotic associations have the potential to impact the relative costs and benefits of symbiosis for hosts and symbionts and the ecological functions of the association, and these impacts are likely to be strongly contingent on factors such as changes in host and symbiont growth dynamics, the capacity for hosts to regulate symbionts and flexibility in host nutritional mode. We will discuss the application of these ideas in detail, where appropriate, in chapters 2-4.

See Figure 1.

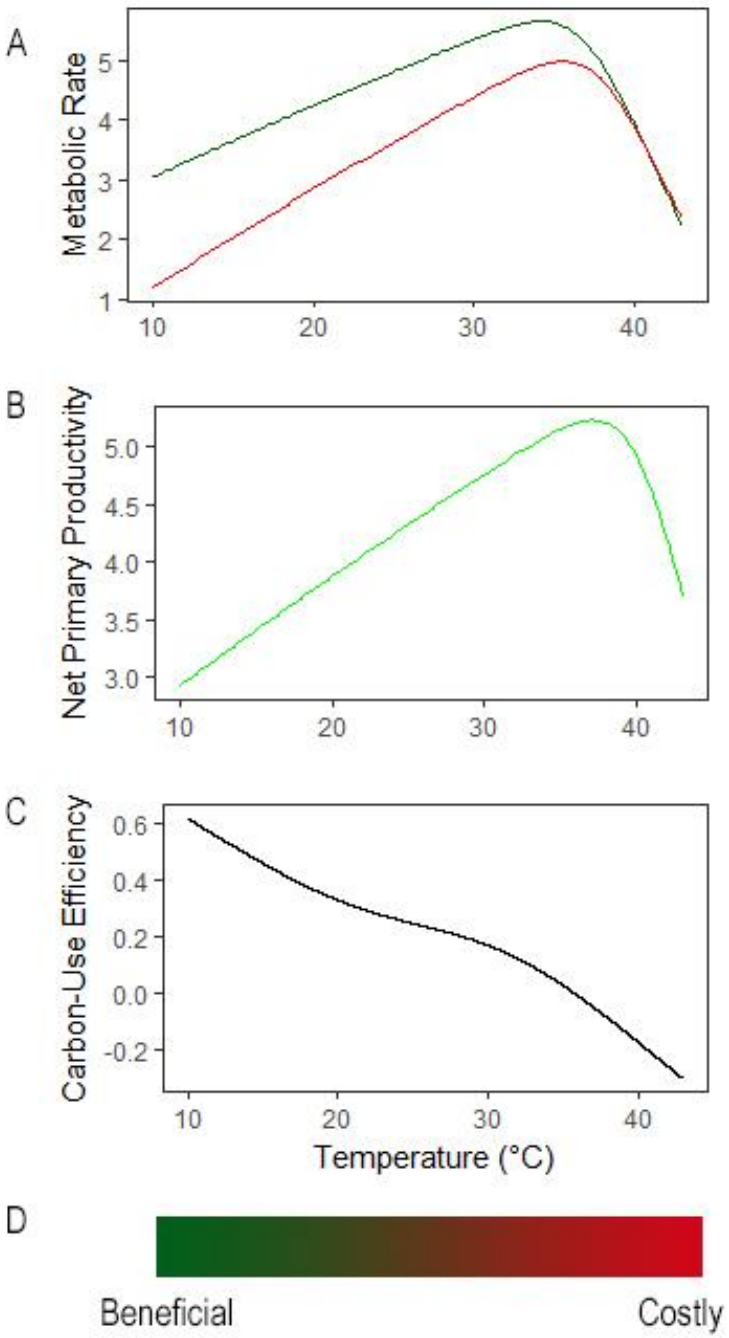


Figure 1: The MTE and photo-symbiosis.

(a) Theoretical metabolic rates in response to temperature. As temperature increases, respiration (R; red) increases more with every degree of warming relative

to photosynthesis (gross primary production; GPP; dark green). Both fluxes follow unimodal responses and approach optima, after which pathological impacts of temperature rapidly drive down rates. (b) Theoretical net primary productivity (NPP) rate in response to temperature. As temperature increases, NPP also follows a unimodal response. NPP = GPP - R (absolute). (c) Theoretical carbon-use efficiency (CUE) in response to temperature. As temperature increases, CUE declines. The shape of this response will vary based on the measured responses of GPP and R. CUE is the theoretical maximum 'fraction' of carbon available for growth and translocation to the host after the respiratory carbon demand has been satisfied. CUE = 1 – R/GPP. Note that although NPP increases with sub-pathological warming, CUE declines; this is because CUE is a relative balance while NPP is an absolute rate. (d) Theoretical benefit/cost of symbiosis for hosts as a result of CUE. As temperature increases and CUE declines, the photosynthetic benefit of harbouring symbionts is expected to be eroded as a result of the falling fraction of carbon available for translocation/metabolite trade.

Research questions addressed and key findings

In chapter 2, owing to the potential importance of CUE in photo-symbiosis (see Box 1), we investigate whether CUE responds as is typical of free-living algae in symbionts or whether co-adaptation to the symbiotic environment could impact this thermal response as a direct consequence of specialisation on different nitrogen sources. We test this idea by examining the impacts of different nitrogen sources on the relative thermal sensitivities of respiration and photosynthesis in symbiotic and free-living *Chlorella spp*. We found that, in free-living *Chlorella* (*Chlorella vulgaris*), nitrogen source influenced metabolic thermal responses and the respiratory cost of growth. Glutamine, in contrast to the other nitrogen sources, resulted in the maintenance of CUE with increases in temperature and results in the lowest respiratory cost of growth. We also found that symbionts were unable to grow on nitrate, but their CUE responses when tested 'in situ' (and grown independently on an amino acid-rich medium) matched those of free-living *Chlorella* on glutamine. Taken together, these observations suggest that the adaptation to symbiosis (via nitrate assimilation degradation and specialisation on amino acids such as glutamine) produces atypical metabolic thermal responses in photo-symbiotic algae.

In chapter 3, we assess the role of metabolism and growth in determining the thermal responses of the widespread and abundant *P. bursaria-Chlorella spp*. photo-symbiosis, and test the predictions that warming can a) cause metabolic disruption that is likely to impact the metabolite trade that underpins the association and b) disrupt the growth dynamics of hosts and symbionts, both/either of which could drive the loss of symbionts and a rise in heterotrophic feeding. In line with this hypothesis,

we found that both warming and cooling drove increases in bacterivory and reduced symbiont abundances within hosts, suggesting that departures from the thermal growth optimum for symbionts can cause the 'bleaching' of, and increased heterotrophy in, widespread photo-symbiotic ciliates. Thus, the thermal growth responses of autotrophs will likely be critical in assessing the responses of their associated photo-symbioses to warming.

In chapter 4, we use 295 days (~10.5 months and ~21 generations) of controlled temperature change to investigate the evolutionary responses of the photo-symbiosis to long-term experimental warming (+5°C). Following these temperature regimes, we found that there was an increase in the thermal optima for holobiont (i.e. symbiotic host with intracellular symbionts living inside) growth with no change in maximum growth rate. Concurrently, symbionts isolated from the long-term warming treatment were able to grow on inorganic nitrogen sources, suggesting that they had gained/re-gained the capacity for free-living growth. Thus, we found that warming produced an adaptive growth response in holobionts but also appeared to drive the evolution of increased autonomy in symbionts, suggesting that two disparate evolutionary trajectories can be simultaneously followed by photo-symbioses. In the context of the ecological role of photo-symbioses, we also found that warm-adapted holobionts had significantly lower rates of primary production (strikingly, net primary production rates approached ~0), suggesting that the ecological function of photo-symbioses can change substantially with warming.

Box 3: Definitions and abbreviations

R: Respiration

GPP: Gross primary production; photosynthesis

NPP: Net primary production; i.e. GPP - R (absolute).

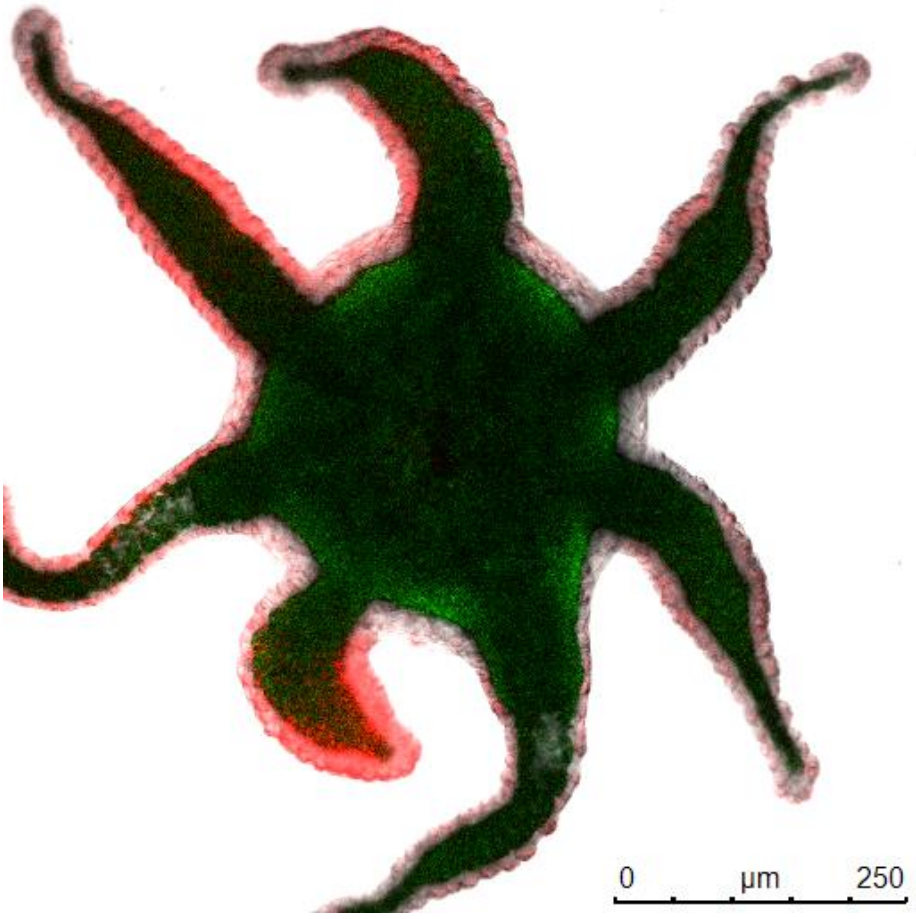
CUE: Carbon-use efficiency; i.e. 1 – R/GPP; the relative fraction of fixed carbon available after respiratory consumption

MTE: The metabolic theory of ecology, which posits that the rates of metabolism are fundamental in shaping a wide suite of patterns in ecology (Brown et al. 2004)

Holobiont: The collective term for symbionts and hosts; i.e. hosts that contain symbionts. In this thesis, we will not use this term to necessarily imply that co-evolution is taking place

Chapter 2

The thermal response of carbon-use efficiency in photo-symbionts depends on nitrogen assimilation



Abstract

How co-adaptation to the symbiotic milieu influences responses to environmental selection pressures such as warming represents an ongoing open research question. One such example of co-adaptation that has recently been identified in the widespread and important association, photo-symbiosis, is the degradation of the nitrate assimilation pathway. Here, symbionts lose access to nitrate and specialise on host-provided compounds (e.g. glutamine). Notably, there are likely to be different energetic costs associated with the assimilation of these nitrogen sources, and this will likely impact the responses of key metabolic processes in symbionts to temperature. Consequently, we predicted that the thermal responses of free-living algae and symbiotic algae would differ as a direct consequence of specialisation on different nitrogen sources. Here, we test this idea by examining the impacts of different nitrogen sources on the relative thermal sensitivities of respiration and photosynthesis in symbiotic and free-living Chlorella spp. We found that, in free-living Chlorella (Chlorella vulgaris), nitrogen source influenced metabolic thermal responses and the respiratory cost of growth. Glutamine, in contrast to the other nitrogen sources, resulted in the maintenance of carbon-use efficiency (CUE) with increases in temperature and results in the lowest respiratory cost of growth. We also found that symbionts were unable to grow on nitrate, but their CUE responses when tested 'in situ' (and grown independently on an amino acid-rich medium) matched those of free-living Chlorella on glutamine. Taken together, these observations suggest that the adaptation to symbiosis (via nitrate assimilation degradation and specialisation on amino acids such as glutamine) produces atypical metabolic thermal responses in photo-symbiotic algae. This work also suggests that

the benefit of nitrogen specialisation in symbionts is likely to be highly dependent on temperature, helping to explain why metabolic integration is apparently not always selected for in nature and indicating that the abiotic environment can determine the extent to which co-dependency will be favoured.

Introduction

Living in symbiosis incurs the adaptation of hosts and/or symbionts to the symbiotic milieu that can lead to co-adaptations and varying degrees of dependency. On one side of this spectrum, classic examples of 'extreme dependency' (where organisms have become integrated; Fisher et al. 2017) include the endosymbiosis between an alphaproteobacterium and the proto-eukaryote that led to the acquisition of mitochondria and the emergence of eukaryotes (Sagan 1967; for a recent review see Roger et al. 2017), the endosymbiosis between eukaryotes and cyanobacteria that led to the development of plastids (Dyall, Brown & Johnson, 2004) and the independent endosymbiotic event exemplified by the amoeba *Paulinella chromatophora* and its chromatophore, which is derived from the cyanobacterium *Synechococcus* (Marin, Nowack & Melkonian, 2005). However, symbiotic organisms are clearly also embedded within the abiotic environment, which demands its own environmental adaptation (Hoffman & Carla, 2011). How symbiotic co-adaptation and responses to the environment interact remains an open research question (Northfield & Ives, 2013; Yoder & Nuismer, 2010).

Photo-symbioses, in which unicellular autotrophs reside within heterotrophic hosts, are a classic example of a widespread and ecologically important symbiosis (Minter et al. 2018) and represent a useful model system for understanding the interaction between co-adaptation and environmental responses in symbiosis. One hallmark of strong co-adaptation between hosts and symbionts is genomic integration, whereby symbiotic organisms have become subject to genome reductions as a result of gene losses or lateral transfers between partners (Fisher et al. 2017). Symbiotic *Chlorella*

spp. found in *P. bursaria* generally cannot survive in the natural environment (since they are moderately-to-highly dependent on host provisions and require hosts for protection from viruses in a natural setting; Hoshina & Imarmura, 2009). As a result, Hoshina & Imarmura, 2009 have suggested that the *P. bursaria-Chlorella spp*. association is likely to be between the 'persistent symbiont' phase (i.e. featuring a symbiont that is normally unable to live autonomously) and symbiont genome reduction phase, where symbiont genes for processes that allow for autonomy may be lost from symbionts (some of which may potentially be transferred to hosts via lateral gene transfer). Exemplifying recently-identified specific symbiont gene losses in *Chlorella* symbionts, it is now known that the capacity for nitrate assimilation in symbiotic *Chlorella spp*. has been lost at least three times: twice in *Chlorella* symbiotic with *P. bursaria* (Kato & Imamura, 2009; Kamako et al. 2005) and once in *Chlorella* symbiotic with *Hydra viridissima* (Hamada et al. 2018). These degradations are independent evolutionary events and represent co-adaptations (Kamako et al. 2005; Hamada et al. 2018; Kato & Imamura, 2009), resulting in dependency of symbionts on host-derived nitrogen.

How could this co-adaptation impact environmental responses, and vice versa? There are likely to be different energetic costs associated with the use of different nitrogen sources (Sanz-Luque et al. 2015); indeed, conversions between nitrogen compounds are among the most energy-demanding reactions in biochemistry (Bloom, 2014). These metabolic costs are likely to be highly dependent on temperature; a fundamental determinant of metabolic rate (Brown et al. 2004). Consequently, it is reasonable to expect that the thermal responses of free-living algae and symbiotic algae (that have become dependent on host-derived nitrogen

sources such as glutamine; Albers et al. 1982; He et al. 2019) could differ, potentially representing one tractable outcome of the interaction between co-adaptation and environment in photo-symbiosis. Here, we test this idea by addressing how changes in nitrogen metabolism associated with symbiosis formation impact broader metabolic and growth responses to temperature. To do so, we examine the impacts of different nitrogen sources on the relative thermal sensitivities of respiration and photosynthesis in symbiotic and free-living *Chlorella spp*.

Methods

Culture conditions and nitrogen sources

Chlorella vulgaris (Sciento, Manchester, UK) cultures were kept in the appropriate medium (Bold's Basal Medium with the appropriate nitrogen source at equimolar concentrations) under a 12:12 h light/dark cycle (~100 µmol PAR m-2 s-1) at the appropriate temperature according to the specific procedure detailed in each section below. The nitrogen sources we used were: nitrate ions, ammonium or glutamine. They were incorporated into the growth medium by dissolving the appropriate compound (sodium nitrate for nitrate ions or ammonium or glutamine) in Bold's Basal Medium (BBM) that did not contain a nitrogen source on its own. The concentration of nitrogen in each medium was controlled by adjusting the volume of compound dissolved in each such that the molarity matched the standard molarity of nitrogen in BBM (see BBM recipe documentation available at CCAP, provided in references). The nitrogen sources were selected since they 'enter' the nitrogen assimilation pathway at different stages (nitrate enters at the basal stage, ammonium later, and

glutamine last; Sanz-Luque et al. 2015), because glutamine is a likely candidate for host provisioning in the case of closely-related symbiotic *Chlorella* species, and because they allowed us to test the impacts of nitrate assimilation deficiency compared to ammonium and glutamine.

Autonomous symbiotic *Chlorella spp*. cultures were established by first washing a *P. bursaria* culture over 10µm filter papers with ~50mL Volvic to remove bacteria. Washed cells were then sonicated (using 3 pulses of 10s at 90% amplitude over ice) to rupture host cell membranes and release the symbiotic algae. Symbionts were then tested for their ability to grow in 3 alternative media: 1) BBM with the standard nitrate nitrogen source. 2) BBM supplemented with a cell-free lysis of *P. bursaria* (collected by sonicating *P. bursaria* cells as above and filtering the lysed cultures and passing through 10µm filter papers where the filtrate was collected). 3) BBM supplemented with bacto-peptone at 1g/L. Bacto-peptone was chosen since it provides a suite of nitrogen compounds including amino acids, which are known to be used by *Chlorella* symbionts that are dependent on hosts for nitrogen provision (e.g. Kato & Imamura, 2009). This assessment was based on measuring growth rate on each medium following the methods for growth rate detailed above. Following successful growth using bacto-peptone, symbionts were grown and transferred intro fresh medium 3 times before being assessed for metabolic responses (see below).

Growth measurements

Three replicate cultures were established for each of 3 temperature treatments (19, 25 and 31°C) for each nitrogen source. Cultures were incubated for 4 days and sub-samples were taken at the start and end of the incubation period and abundance/density was enumerated via flow cytometry. Growth rate was calculated using the decadic logarithm of cell counts and assuming exponential growth using the 'lmList' function in the 'lme4' R package (Bates et al. 2015) in R statistical software (v3.2.0) (R Core Team, 2014).

Metabolic measurements

We measured respiration (R), gross photosynthesis (GPP) and carbon-use efficiency (CUE; 1-R/GPP) for *C. vulgaris* on each nitrogen source across a temperature gradient (13, 19, 25 and 31°C) using 3 biological replicate cultures (i.e. 3 cultures at each temperature on each source) taken from a single reference long-term stock (historically grown on nitrate, as is typical of free-living *Chlorella*) kept at 20°C.

We measured net primary production (NPP) (via rate of change of oxygen concentration at different light intensities) and respiration (R) (via rate of change of oxygen concentration in the dark) in 1mL aliquots of cultures. Culture aliquots were acclimatised to the assay temperature for 30 minutes in the dark. Oxygen evolution measurements were conducted using a Clark-type oxygen electrode (Hansatech Ltd; King's Lynn, UK; Chlorolab2). R was estimated as the rate of change of oxygen

concentration (i.e. via uptake by respiring organisms) in the dark. NPP was measured at increasing light intensities in intervals of 50 μmol^{-1} m^{-2} s^{-1} (PFD) up to 200 PFD, and then in intervals of 100 PFD up to 1000 PDF, and finally at 1200, 1500 and 1800 PDF. This yielded a photosynthesis irradiance curve (PI) at each assay temperature; these curves were fitted to a photoinhibition model (Platt *et al*. 1990) using non-linear least squares regression (following the statistical methods described previously in Padfield *et al*. 2016). The maximum oxygen evolution in the light (i.e. at the optimum light intensity) was taken as the maximum NPP (Pmax). We used Pmax to control for any potential interactions between light intensity and temperature in measuring the thermal response of NPP. Gross primary production (GPP) was then estimated as:

$$GPP = Pmax + R \text{ (absolute)}$$

CUE was then estimated as:

$$CUE = 1 - R/GPP$$

We also tested one stock (using 3 technical replicates) of autonomous symbionts (see above) and one of 'freshly-liberated/in-situ symbionts' across a 12-temperature gradient such that we could fit Sharpe-Schoolfield equations to the data (see below), yielding activation energies (E_a), thus enabling comparisons of symbiont metabolism with previously published data and to our findings in Chapter 3. 'In situ' symbionts were measured by sonicating a *P. bursaria* culture during mid-log growth phase and taking the same measurements immediately.

Responses of GPP and R to temperature for ‘in-situ’ and autonomous symbionts were fitted to a modified Sharpe-Schoolfield equation for high temperature inactivation using non-linear least squares regression; fits were determined using the ‘nls_multstart’ function in the ‘nls.multstart’ package (Padfield & Matheson, 2018) in R statistical software. This package compares AIC values to identify the parameter set, drawn from a uniform distribution, which best characterises the data. The goodness of fit of the selected models were examined graphically and via assessment of pseudo-R2 values. These parameter sets yielded activation energies (E_a; pre-optimal gradients that are frequently used to characterise and compare the temperature sensitivities of metabolic rates; Padfield *et al*. 2016) for both respiration and photosynthesis (GPP), which could be compared.

In addition, it is sometimes useful to ascribe respiration to its two contrasting ‘outputs’: growth and maintenance processes (e.g. Amthor, 1988). Thus, we calculated a simple parameter for the relative metabolic ‘cost’ associated with growth:

Respiratory cost of respiration (R/μ) = Respiration / Growth Rate

We measured growth rates on each nitrogen source at each temperature for *C. vulgaris* following the growth methods outlined above and subsequently calculated R/μ at each temperature, using the mean R values calculated for each temperature for each stock. This parameter provides an estimate of the efficiency with which respiration leads to growth output, as opposed to maintenance processes (i.e. low R/μ implies high efficiency and a low proportion of respiration that is feeding into maintenance processes).

Results

Free-living Chlorella (C. vulgaris)

- *Metabolism and Carbon-use Efficiency*

We measured R and GPP on the 3 alternative nitrogen sources across a temperature gradient to incorporate the metabolic impacts of temperature change. We then modelled R and GPP independently using linear mixed effects models with a random effect of replicate nested within nitrogen source. Both R and GPP increased with temperature on all 3 sources (Figure 1a); nitrogen source significantly affected the temperature response of GPP (L.Ratio=8.267, df=2,8, p=0.0160; Table S1). Through model selection (Table S1), we dropped the interaction between temperature and nitrogen source and the main effect of nitrogen source from our model for R; here, only temperature remained in the model and thus temperature alone determined R (L.Ratio=8.267, df=1,4, p<0.0001).

Using our measured R and GPP values, we calculated carbon-use efficiency (CUE) across this temperature gradient for each nitrogen source. In our CUE linear mixed effect model, nitrogen source significantly impacted the thermal response of CUE (L.Ratio=12.113, df=2,8, p=0.0023; Table S1). We observed declines in CUE with warming on nitrate and ammonium and a shallow increase with warming on glutamine (Figure 1b; Table S2).

- *Respiratory cost of growth*

We also estimated the respiratory cost of growth (R/μ) on the 3 alternative nitrogen sources each at 3 temperatures to incorporate the metabolic impacts of temperature change. Nitrogen source significantly explained R/μ ($F=36.4$, $df=2,25$, $p<0.0001$; Figure 2) but temperature did not interact with nitrogen source and thus the interaction term was dropped from our linear model (Table S3). Tukey's multiple comparisons of means revealed significant differences between R/μ on glutamine and the other sources (ammonium and nitrate) (Table S4). R/μ was significantly lower across all temperatures on glutamine (compared to nitrate and ammonium). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons.

Symbiotic Chlorella

- *Chlorella symbiont growth on nitrate*

We isolated *Chlorella* symbionts from within their *P. bursaria* hosts and tested their capacity for growth on different media, where one contained nitrates as the sole nitrogen source, another bacto-peptone as the nitrogen source (i.e. various amino acids and nitrogen compounds) and a final containing a cell-free extract from symbiotic hosts. The symbionts were unable to grow on the nitrate medium, appeared to grow slowly on the host extract and grew rapidly on bacto-peptone.

Medium significantly explained growth rate ($F=509.31$, $df=2,8$, $p<0.0001$; Figure 3; Table S3). Tukey's multiple comparisons of means revealed significant differences in growth rate between all media (Table S4). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons.

We also tested for significant differences between the growth rates on each medium and zero. T-tests revealed that significant differences were obtained for the nitrate source ($t=-21.346$, $df=2$, $p=0.002188$) and bacto-peptone source ($t=36.545$, $df=2$, $p=0.0007479$) but not the cell-free host extract source ($t=3.5038$, $df=2$, $p=0.07269$), indicating that growth rate on the nitrate source was negative, on the bacto-peptone source was positive and on the host extract source was not significantly different to zero.

- *Metabolism and Carbon-use Efficiency*

We measured R and GPP across a 12-temperature gradient to incorporate the metabolic impacts of temperature change. We then modelled R and GPP independently using modified Sharpe-Schoolfield equations, yielding E_a values for the two fluxes. In 'freshly-liberated' symbionts, representing a scenario as close to 'In situ' as possible (i.e. immediately following cell lysis), R and GPP had similar activation energies; $E_a^R = 0.816$ and $E_a^{GPP} = 0.847$ (Figure 4a; Table 1). We also conducted the same measurements on symbionts that had been extracted and then

grown independently of hosts in a medium rich in various nitrogen compounds and amino acids (see Methods). Here, too, R and GPP had similar activation energies; E_a^R = 0.720 and E_a^{GPP} = 0.730 (Figure 5a; Table 1).

CUE (calculated at ≤40°C due to a lack of measurable response at 43°C) did not change with temperature across the full temperature range in freshly-liberated symbionts (F=0.493, df=1,32, p=0.4879; Figure 4b; Table S3) and remained relatively constant below ~30°C in autonomous/independently-grown symbionts (Generalised additive model for a non-linear response; Table S5; Figure 5b).

Discussion

Here, we examine photosymbiosis – an important and widespread association (Minter et al. 2018) – and address how changes in nitrogen metabolism associated with symbiosis formation impact broader metabolic and growth responses to temperature. We predicted that the thermal responses of free-living algae and symbiotic algae would differ as a direct consequence of specialisation on different nitrogen sources. Here, we test this idea by examining the impacts of different nitrogen sources on the relative thermal sensitivities of respiration and photosynthesis in symbiotic and free-living *Chlorella spp*. We found that, in free-living *Chlorella* (*C. vulgaris*), nitrogen source influenced metabolic thermal responses and the respiratory cost of growth. Glutamine, in contrast to the other nitrogen sources, resulted in the maintenance of CUE with increases in temperature and incurred the lowest respiratory cost of growth. We also found that symbionts were unable to grow

on nitrate, but their CUE responses when tested ‘in situ’ (or grown independently on an amino acid-rich medium) matched those of free-living *Chlorella* on glutamine. Taken together, these observations suggest that adaptation to symbiosis (via nitrate assimilation degradation and specialisation on amino acids such as glutamine) produces atypical metabolic thermal responses in photo-symbiotic algae.

First, to investigate the impacts of the assimilation of different nitrogen sources on metabolism, we measured R, GPP and CUE in the free-living *C. vulgaris* – a close ancestor of symbiotic *Chlorella* in *P. bursaria* and *H. viridissima* that carries an intact nitrogen assimilation system (Hoshina & Imarmura, 2009; Sanz-Luque et al. 2015; Syrett, 1956). This allowed us to assess what the metabolic impacts of nitrogen source were likely to be prior to the adaptation to symbiosis via degradation of nitrate assimilation capacity in the symbiotic strains. CUE is a relative ratio of P and R and has been used as a tractable means of estimating the maximum proportion of carbon available for growth processes after accounting for respiratory losses in autonomous algae; this has been shown to be strongly dependent on temperature (Padfield et al. 2016). Thus, it could be useful in a photo-symbiotic context, because it will likely determine the relative volume of photosynthates that would theoretically be available for metabolite trade with hosts. We determined metabolic responses across a temperature gradient, finding that the nitrogen source impacted the thermal response of GPP but nor R; thus, any differences in CUE will be driven by GPP. Strikingly, CUE declined rapidly with temperature on nitrate and ammonium, but not on glutamine (where it was in fact subject to a shallow increase). This suggests that there is a direct, temperature-dependent benefit to CUE of assimilating glutamine (compared to ammonium and nitrate). In the context of photo-symbiosis, this could

suggest that there would theoretically be a temperature-dependent cost incurred by nitrate assimilation, given the option of assimilating glutamine. Similarly, we found that growth of *C. vulgaris* on glutamine incurs a lower respiratory cost (i.e. R/μ is low on glutamine compared to the other sources); this indicates that less respiration was associated with maintenance processes on glutamine, probably reflecting the reduced number of 'steps' that must be sequentially passed in the nitrogen assimilation pathway in order to process the contained nitrogen (Sanz-Luque et al. 2015).

Next, we reconciled our measurements of GPP, R and CUE with symbionts obtained from *P. bursaria*, where genetic and physiological nitrate assimilation deficiency has been shown in a closely-related strain (Kato & Imamura, 2009; Kamako et al. 2005; Hoshina & Imarmura, 2009). As expected, symbionts could not grow autonomously on nitrate, suggesting that they could not assimilate nitrate as a result of the genetic degradation previously reported (Kato & Imamura, 2009; Kamako et al. 2005). Strikingly, we found that the temperature-sensitivity of GPP in these symbionts was roughly twice that which is reported in the literature for communities and ecosystems (Allen et al. 2005; Lopez-Urrutia et al. 2006; Yvon-Durocher et al. 2010; Regaudie-De-Gioux & Duarte 2012), while the temperature-sensitivity of R was only comparatively slightly higher (Allen et al. 2005; Yvon-Durocher et al. 2012). However, recent work suggests that the activation energies of GPP and R could be different when measured in free-living phytoplankton compared to the communities and ecosystems previously studied (Allen et al. 2005; Lopez-Urrutia et al. 2006; Yvon-Durocher et al. 2010; 2012; Regaudie-De-Gioux & Duarte 2012); E_a^{GPP} was higher at 0.74eV and E_a^{R} at 1.07eV when pooled across 18 diverse marine

phytoplankton (Barton et al. 2020), in agreement with other recent studies on free-living algae (Padfield et al. 2016; Schaum et al. 2017). More work is clearly required, yet in light of these data, the thermal sensitivity of GPP appears to be higher and that of R appears to be lower than expected in the freshly liberated *P. bursaria* symbiont (E_a^R = 0.816 and E_a^{GPP} = 0.847; Table 1) when compared with free-living algae. The thermal sensitivity of GPP appears to be similar while that of R is also lower when considering autonomous symbionts (E_a^R = 0.720 and E_a^{GPP} = 0.730). The similar thermal sensitivities of R and GPP meant that symbiont CUE remained largely invariant with temperatures under ~30°C, in stark contrast with previously published data on free-living algae (Barton et al. 2020; Padfield et al. 2016; Schaum et al. 2017) as well as ecosystems and communities (Allen et al. 2005; Lopez-Urrutia et al. 2006; Yvon-Durocher et al. 2010; 2012; Regaudie-De-Gioux & Duarte 2012). Our metabolic and growth measurements in *C. vulgaris* suggest reasons for these observed differences in the metabolic thermal sensitivities: E_a^{GPP} could potentially be increased and maintenance R could be reduced (theoretically reducing E_a^R, although we did not detect this in our *C. vulgaris* measurements here; Barton et al. 2018) in symbionts via the use of glutamine as a nitrogen source.

We suspected that the thermal responses of free-living algae and symbiotic algae (that have become dependent on host-derived nitrogen sources such as glutamine; Albers et al. 1982; He et al. 2019) could differ, potentially representing one tractable outcome of the interaction between adaptation to symbiosis and to the abiotic environment. Our data support this idea: the co-adaptation exemplified by metabolic integration in photo-symbiosis, which has driven nitrogen specialisation in symbionts, appears to have fundamentally changed the metabolic responses of the symbionts to

warming. In agreement with recent suggestions by Kato & Imamura, 2009 and Hamada et al. 2018, the loss of nitrate assimilation in *Chlorella* symbionts is likely delivering a tractable benefit in the symbiotic milieu. Specifically: relative to nitrate, glutamine assimilation a) generates a temperature-dependent CUE increase and b) reduces the respiratory maintenance cost. Thus, the assimilation of key nutrients such as nitrogen could support the progression toward extreme host dependence on symbionts, an important problem for evolutionary biologists (e.g. Fisher et al. 2017) and could likewise drive symbiont dependence on hosts (e.g. Hamada et al. 2018). Notably, our findings suggest that the metabolic benefit of glutamine use over nitrate is likely to become stronger with warming. Thus, factors such as temperature should be critical in understanding the conditions under which metabolic co-dependency is favoured and when it is not. Indeed, a complicated pattern appears to be emerging in *P. bursaria* symbioses, whereby the large assortment of strains of hosts and symbionts display varying degrees of dependency on one another (Minter et al. 2018). Furthermore, in general, there are numerous symbionts in other photo-symbioses that have not lost the ability to assimilate nitrate, where it may be adaptive to retain nitrate functionality (Hamada et al. 2018). These complex phenomena may better be investigated by first understanding when co-dependency is likely to be strongly selected for; here, we show that temperature is likely an important factor as a result of its role in determining the benefits likely provided by nitrogen source specialisation and co-adaptation in photo-symbiosis.

Conclusion

The capacity for nitrate assimilation in symbiotic *Chlorella* has been lost at least three times: twice in *Chlorella* symbiotic with *P. bursaria* (Kato & Imamura, 2009; Kamako et al. 2005) and once in *Chlorella* symbiotic with *H. viridissima* (Hamada et al. 2018). These losses of nitrate assimilation are independent evolutionary events (Kamako et al. 2005; Hamada et al. 2018; Kato & Imamura, 2009) and represent co-adaptations resulting in dependency of symbionts on host-derived nitrogen. Here we show that, as a consequence of this shift in symbiont nitrogen metabolism, the metabolic thermal responses of symbionts differ substantially from those of free-living algae.

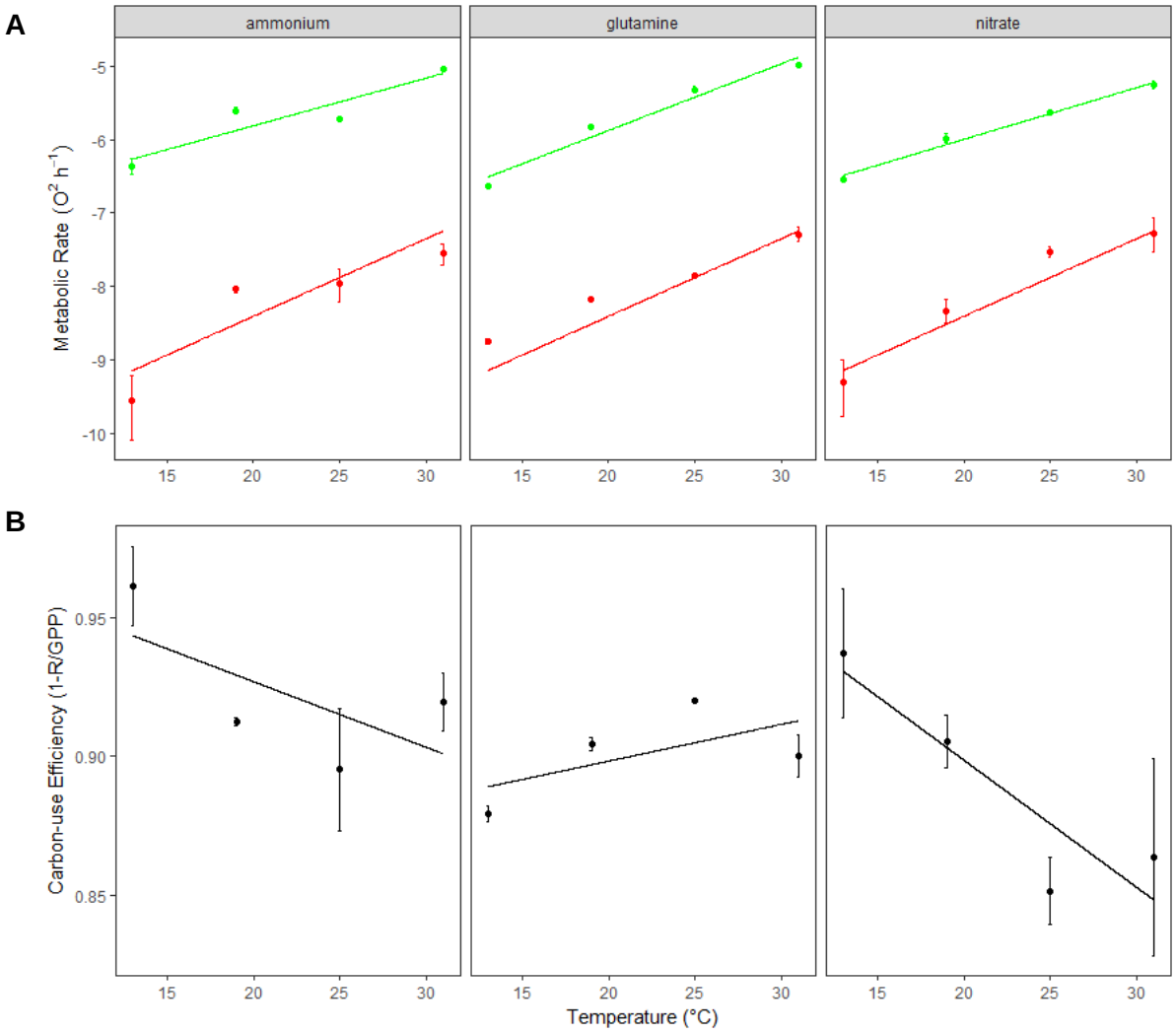


Figure 1: *Chlorella vulgaris* metabolic flux and carbon-use efficiency on different nitrogen sources across a temperature gradient

Green = GPP; Red = R. Lines represent fitted linear models. Number of replicates = 3. Points represent means ±SE.

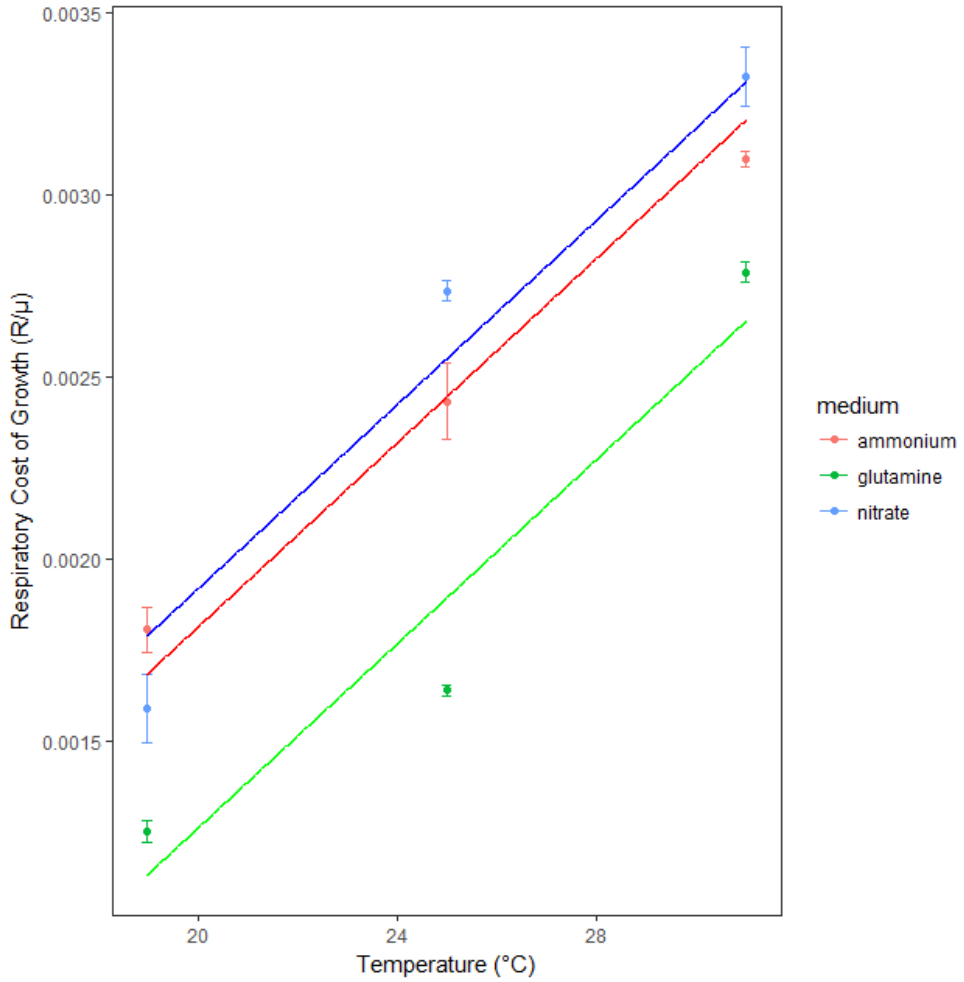


Figure 2: *Chlorella vulgaris* respiratory cost of growth on different nitrogen sources across a temperature gradient

Lines represent fitted linear model to data. Points represent means ±SE. Number of replicates = 3.

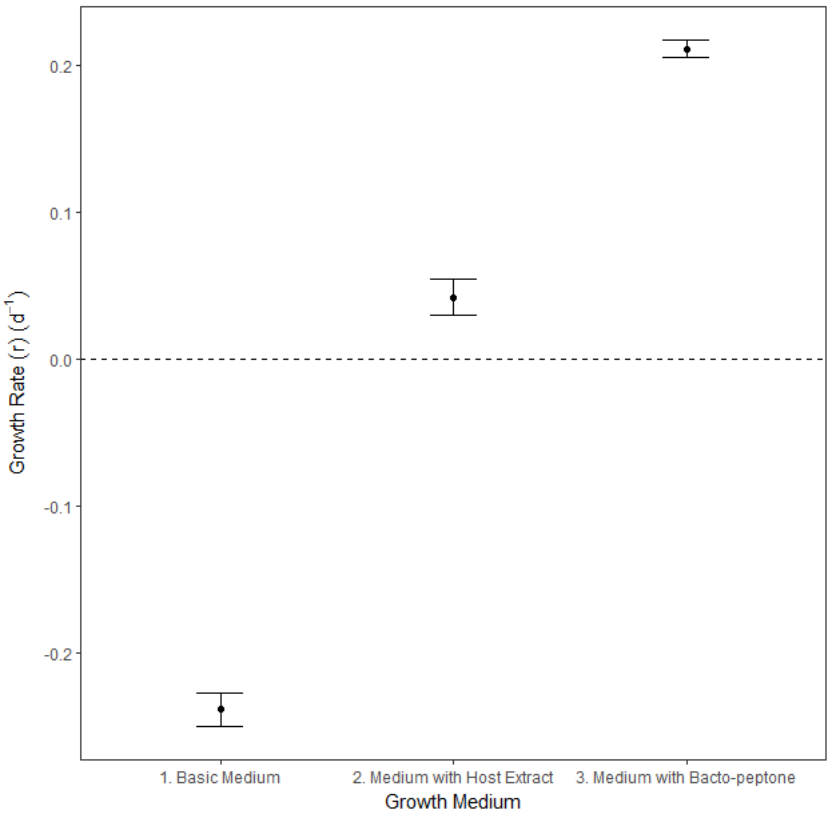


Figure 3: Isolated symbiont growth in different media

Groups: 1) containing nitrate, 2) containing host extract; 3) containing bacto-peptone (various amino acids and nitrogen compounds). Points represent means ±SE. Number of replicates = 3. Means were also tested for significant difference from zero (see Results).

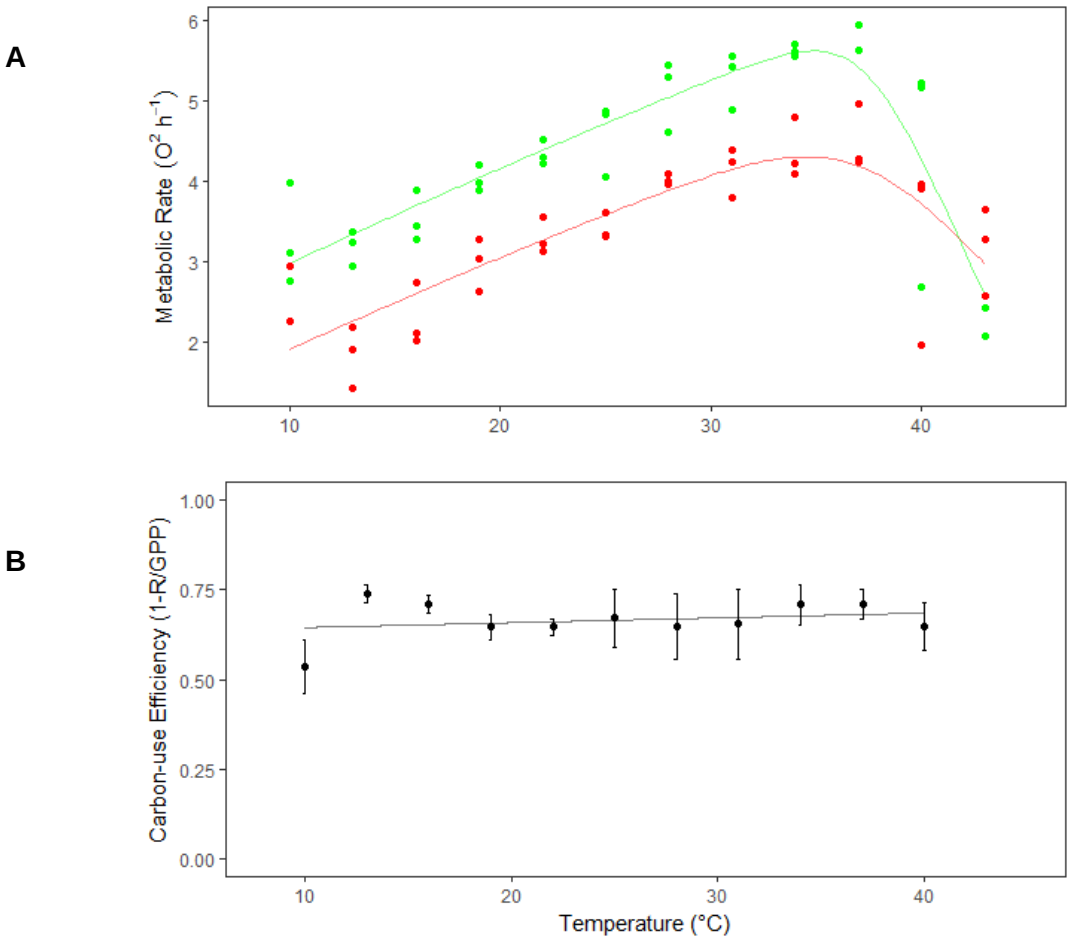


Figure 4: 'In situ' symbiont metabolic flux and carbon-use efficiency across a temperature gradient

Green = GPP; Red = R. Lines in metabolic rate panel represent fitted Sharpe-Schoolfield equations (see Methods and Results). Line in CUE panel represents fitted linear model to data. Number of replicates = 3. Points represent means ±SE in CUE panel.

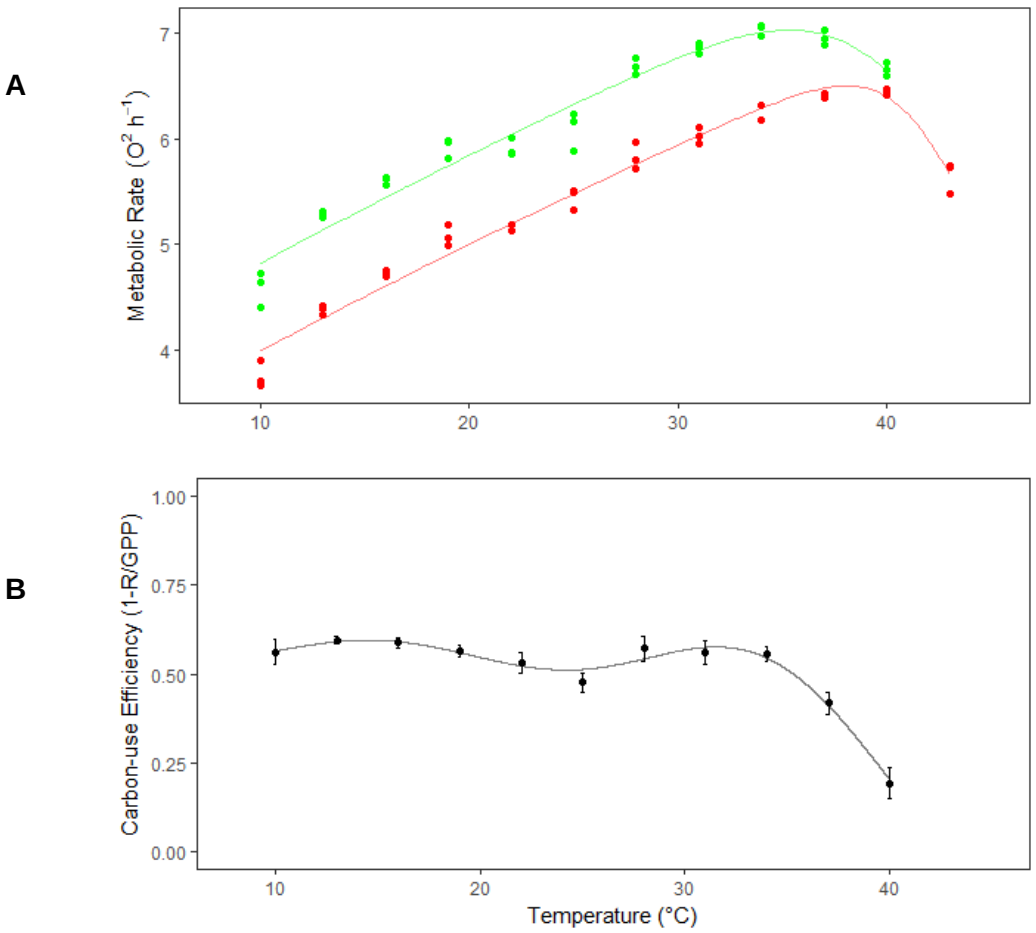


Figure 5: Autonomous symbiont metabolic flux and carbon-use efficiency across a temperature gradient

Green = GPP; Red = R. Lines in metabolic rate panel represent fitted Sharpe-Schoolfield equations (see Methods and Results). Line in CUE panel represents fitted GAM to data. Number of replicates = 3. Points represent means ±SE in CUE panel.

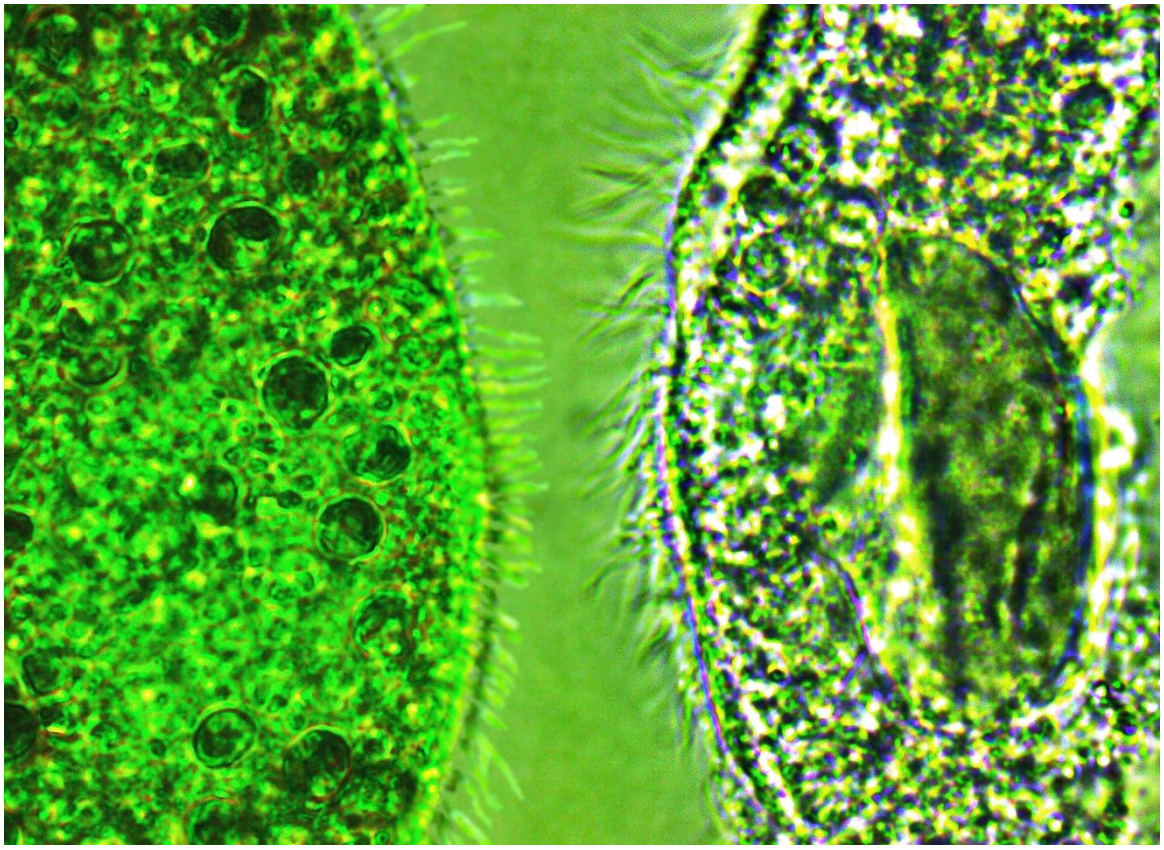
Type	*Flux*	E_a	*std.error*	*statistic*	*p.value*
Autonomous	GPP	0.730	0.0441	16.5	2.60e-16
Autonomous	R	0.720	0.0243	29.6	7.86e-25
'In situ'	GPP	0.847	0.0975	8.68	8.35e-10
'In situ'	R	0.816	0.120	6.81	1.05e- 7

Table 1: Sharpe-Schoolfield curve estimates for metabolic activation energies

Activation values (E_a; eV) derived from the fitted Sharpe-Schoolfield curves to autonomous and 'in-situ' symbiont metabolic thermal responses. These values represent the sensitivity of the flux (photosynthesis; GPP or respiration; R) to temperature and can usefully compared to previously published values (e.g. Padfield et al. 2016).

Chapter 3

‘Ciliate bleaching’: The thermal niche of algal symbionts constrains symbiont load within photo-symbiotic ciliates



Abstract

Photo-symbioses, in which unicellular autotrophs reside within heterotrophic hosts, are widespread and important ecological interactions in terms of global biomass, species diversity and primary production. These associations are seemingly highly sensitive to temperature and are under threat from environmental warming. While specific impacts of warming that can lead to the loss of symbionts from hosts – particularly in the coral-zooxanthellae interaction – are thoroughly documented, fundamental mechanisms that result in symbiont loss ('bleaching') and the breakdown of photo-symbiosis are not well understood. Here, we assess the role of metabolism and growth in these responses in a widespread and abundant photo-symbiotic ciliate, and test the predictions that warming can a) cause metabolic disruption that is likely to impact the metabolite trade that underpins the association and b) disrupt the growth dynamics of hosts and symbionts, driving the loss of symbionts and a rise in heterotrophic feeding. Intriguingly, we found that both warming and cooling drove increases in bacterivory and reduced symbiont abundances within hosts, suggesting that departures from the thermal growth optimum for symbionts (and not metabolic thermal responses per se) can cause the 'bleaching' of, and increased heterotrophy in, widespread photo-symbiotic ciliates. Thus, the thermal growth responses of autotrophs will likely be critical in assessing the responses of their associated photo-symbioses to warming.

Introduction

While the impacts of recent climate change on the physiology, life history and spatial distributions of organisms spanning the tree of life are well reported, there is growing concern that global warming will also impact ecological interactions between species (e.g. Walther *et al*. 2002; Tylianakis *et al*. 2008; Walther, 2010), potentially destabilising food webs and disrupting ecosystem functioning on a large scale. Such impacts are arguably likely to be particularly important in the case of mutualisms, since many such associations (e.g. photo-symbioses, plants with mycorrhizal fungi and legumes with nitrogen-fixing bacteria) underpin critical ecosystem functions (reviewed in Kiers *et al*. 2010).

The archetypal example of warming-induced mutualism breakdown is the coral-zooxanthellae symbiosis that underpins coral reef ecosystems, where temperature increase can lead to the stress-associated expulsion of symbionts from their hosts (e.g. Weis, 2008). Here, warming can drive a reduction in symbiont numbers through a disruption of symbiont photosynthesis by a number of chemical and mechanical stressors. However, coral-symbiont associations are representative of a broader group of symbioses in which photosynthetic algae reside within heterotrophic hosts (i.e. photo-symbioses). Many such associations involve unicellular algae residing within unicellular hosts; these are comparatively poorly studied, but are now recognized to be highly abundant and diverse in aquatic ecosystems (Sonntag *et al*. 2011; Summerer *et al*. 2008; Sanders, 1991, 2011; Decelle, Colin & Foster, 2015). Indeed, photo-symbioses are responsible for approximately one half of all marine primary production (Baldauf, 2008) and, when considered alongside photo-

mixotrophs, can often dominate ecosystem bacterivory (Berninger *et al*. 1992; Unrein *et al*. 2007; Zubkov & Tarran, 2008; Hartmann *et al*. 2012). Consequently, understanding the responses of unicellular photosymbioses to warming is likely to be important in understanding the broader ecological impacts of environmental warming on aquatic ecosystems.

While extensive research has described a multitude of specific physiological and ecological impacts of warming on photo-symbiosis, particularly in corals (e.g. Lesser 2004, 2011, 2013; Correa & Baker, 2011; Weis, 2008, 2010), ecologists lack a general predictive framework for the impacts of temperature on photo-symbiotic associations (Bailly *et al*. 2014) and little is known about the likely responses of the numerous microbial photo-symbioses to warming. In this context, the metabolic theory of ecology (MTE), which posits that rates of metabolism are fundamental in shaping a wide suite of patterns in ecology (Brown *et al*. 2004), could be useful. From a metabolic perspective, respiration is more temperature-sensitive than photosynthesis (e.g. López-Urrutia *et al*. 2006; Anderson-Teixeira *et al*. 2011; Yvon-Durocher *et al*. 2010, 2012); this can directly reduce the capacity for autotrophy in ecological systems with warming (e.g. Yvon-Durocher *et al*. 2010; Wilken *et al.* 2013). Mechanistically, an increasing proportion of carbon fixed by photosynthesis is expected to be respired at higher temperatures, reducing the carbon fraction available for growth (termed carbon-use efficiency; CUE); this expectation has been born out by empirical measurements in a wide suite of free-living algae (Padfield *et al*. 2016; Barton et al. 2020). If CUE also declines in photo-symbionts, it is likely that the metabolite trade that underpins photo-symbioses (see Johnson, 2011 for a review) will be disrupted, since a lower fraction of the produced photosynthates will

be available for translocation to hosts, and that autotrophic growth capacity at higher temperatures will be constrained (Barton et al. 2018).

Changes in the metabolic rates in photosymbiotic associations have the potential to impact the relative costs and benefits of symbiosis for hosts and symbionts and the ecological functions of the association. These impacts are likely to be strongly contingent on factors such as changes in host and symbiont growth dynamics, the capacity for hosts to regulate symbionts, and flexibility in host nutritional mode. For example, reduced CUE could potentially drive increases in the acquisition of heterotrophic carbon (e.g. by bacterivory rate increases) in photo-symbioses, as has been observed in autonomous mixotrophs that do not carry photosynthetic symbionts (Wilken *et al.* 2013). Such a shift could be exaggerated if hosts down-regulate symbionts in response to declines in the relative 'value' of harbouring symbionts (caused by CUE decline) via the suite of regulatory mechanisms thought to be employed by *P. bursaria* hosts, such as digestion of symbionts (Kodama & Fujishima, 2008), cell cycle regulation (Kadono *et al*. 2004) and nitrogen restriction (Lowe *et al*. 2016 (and see references therein); He et al. 2019) or by photo-symbiotic hosts in general, such as host-regulated supply of inorganic nutrients (reviewed in Davy *et al*. 2012; see Iwai, Fujiwara & Tamura, 2016; Xiang et al. 2020). Alternatively, recent work has shown that symbiont abundance within *P. bursaria* hosts may primarily be maintained by the effects of growth rate of hosts and symbionts (Iwai, Fujiwara & Tamura, 2016); thus, metabolism could constrain symbiont abundance 'directly' by constraining their growth potential at higher temperatures. In addition, the same regulatory mechanisms could also allow hosts to "choose" (e.g. Foster et al. 2017; see Sachs et al. 2004 and Edwards, 2009)

thermally proficient symbionts from within their intracellular populations and/or from populations that may establish in the growth medium, akin to partner ‘shuffling’ (e.g. Jones et al. 2008) and ‘switching’ (e.g. Lewis & Coffroth, 2004) in corals and lichens (Rolshausen et al. 2018), which could negate the requirement for significant changes in overall symbiont abundance.

In summary, MTE predicts that increased temperature results in shifts to heterotrophy and reduced CUE, which potentially disrupts metabolite trade and constrains autotrophic growth. Consequently, we would predict that hosts should increase bacterivory with temperature and that changes in symbiont density will be a simple function of the thermal growth responses of symbionts. In this study, we tested this simple prediction in the laboratory, by characterising the temperature-dependence of metabolism, growth rates, symbiont load and host bacterivory in the *Paramecium bursaria-Chlorella spp*. photo-symbiosis – a widespread and abundant freshwater ciliate-alga association (see Minter *et al*. 2018).

Methods

Culture conditions

Paramecium bursaria is a widespread and abundant freshwater ciliate (see Minter *et al*. 2018 and references therein) that harbours symbiotic *Chlorella spp*. algae. The particular strain (HA1g, National BioResource Project) used in this study was originally isolated in Hirosaki, Japan in 2010, and has been maintained in our

laboratory at 25°C for ~2 years. Intact *P. bursaria* cells (i.e. symbiotic hosts with their intracellular resident symbionts) were maintained in a single 100mL stock culture of protozoan pellet/Volvic medium inoculated with *Serratia marcescens* for 48 hours prior to use to provide an abundance of bacterial prey (*S. marcescens* is a common bacterium that we found to provide effective *P. bursaria* growth in the laboratory). *P. bursaria* cultures were transferred intro fresh bacterized medium every 2 weeks and were kept under a 12:12 h light/dark cycle (~100 µmol PAR m^{-2} s^{-1}).

Isolated symbiont cultures were established by first washing a *P. bursaria* culture over 10µm filter papers with ~50mL Volvic to remove bacteria. Washed cells were then sonicated (using 3 pulses of 10s at 90% amplitude over ice) to rupture host cell membranes and release the symbiotic algae. Symbionts were established in Bold's Basal Medium (BBM) supplemented with bacto-peptone at 1g/L. Symbionts were grown and transferred intro fresh medium 3 times before use in experiments and were kept under the same light and temperature conditions as the *P. bursaria* stock.

Metabolic thermal responses

To characterise the metabolic thermal responses of *P. bursaria*, we measured net primary production (NPP) (via oxygen evolution at different light intensities) and respiration (R) (via oxygen evolution in the dark) in 1mL aliquots of washed (to remove bacteria and any free-living algal symbionts) cultures across a broad range of assay temperatures (10°C – 43°C). Culture aliquots were acclimatised to the assay temperature for 30 minutes in the dark. Oxygen evolution measurements were

conducted using 3 technical replicates (i.e. 3 measurements at each temperature on the same biological stock culture) using a Clark-type oxygen electrode (Hansatech Ltd; King's Lynn, UK; Chlorolab2). R was estimated as the rate of oxygen evolution in the dark. NPP was measured at increasing light intensities in intervals of 50 $\mu mol^{-1}\ m^{-2}\ s^{-1}$ (PFD) up to 200 PFD, and then in intervals of 100 PFD up to 1000 PDF, and finally at 1200, 1500 and 1800 PDF. This yielded a photosynthesis irradiance curve (PI) at each assay temperature; these curves were fitted to a photoinhibition model (Platt *et al*. 1990) using non-linear least squares regression (following the statistical methods described previously in Padfield *et al*. 2016). The maximum oxygen evolution in the light (i.e. at the optimum light intensity) was taken as the maximum NPP (Pmax). We used Pmax to control for any potential interactions between light intensity and temperature in measuring the thermal response of NPP. Gross primary production (GPP) was then estimated as:

$$GPP = Pmax + R\ (absolute)$$

Responses of GPP and R to temperature were then fitted to a modified Sharpe-Schoolfield equation for high temperature inactivation using non-linear least squares regression; fits were determined using the 'nls_multstart' function in the 'nls.multstart' package (Padfield & Matheson, 2018) in R statistical software ((v3.2.0) (R Core Team, 2014)). This package compares AIC values to identify the parameter set, drawn from a uniform distribution, which best characterises the data. The goodness of fit of the selected models were examined graphically and via assessment of pseudo-R2 values. These parameter sets yielded activation energies (E_a; pre-optimal gradients that are frequently used to characterise and compare the

temperature sensitivities of metabolic rates; Padfield *et al*. 2016) for both respiration and photosynthesis (GPP), which could be compared. CUE was estimated as:

$$CUE = 1 – R/GPP$$

CUE estimates were calculated for each assay temperature and were fit to a general additive model (GAM) to allow for a non-linear response using the ‘gam’ function in the ‘mgcv’ package (Wood, 2006) in R statistical software, where temperature was the explanatory variable and CUE the dependent variable.

Ecological thermal responses

We assessed the thermal responses of symbiont density within hosts, growth rates in holobionts and independently growing symbionts, and host bacterivory across a range of temperatures between 15°C and 35°C (15, 20, 25, 30 and 35°C).

- *Symbiont density measurements*

Symbiont density within hosts was estimated by sonicating a *P. bursaria* stock kept at each of our 5 assay temperatures during mid-log growth phase (using 3 pulses of 10s at 90% amplitude over ice) that was allocated into 3 replicate test cultures (each of 10mL) in order to rupture host cell membranes; we enumerated the resultant released symbiotic algal cells using flow cytometry (BD Accuri C6; BD). Flow cytometry involved simply passing these lysed cultures through a flow cytometer where the number of algae detected in a known volume (10µL) of the sample was

based on chlorophyll fluorescence while the culture was penetrated by a laser. The system was first calibrated as recommended and detailed by the manufacturer and negative controls (i.e. where growth medium alone was run through the flow cytometer) were first compared with algal cultures in trial runs to confirm that isolated algae in our experiments could be successfully detected and counted.

Symbiont counts were adjusted for the density of *P. bursaria* hosts by dividing the number of counted algae by the number of hosts (enumerated via imageJ analysis using the 'blur' followed by 'Find Maxima' base functions (Schindelin *et al*. 2012); the number of hosts lysed per replicate was typically ~1000) of fluorescence microscopy images at 10x magnification (Leica TCS SP8; Leica Microsystems; Wetzlar, Germany)). Symbiont population sizes per host were also divided by mean cell volume estimates to control for changes in host cell volume. Estimates of *P. bursaria* cell volume (μm^3) were based on manual measurements of cell length and width. Volume was calculated assuming that cells were prolate spheroids ($Volume = 4/3\pi a^2 c$; where a and c are the polar radii; the number of hosts measured per replicate was 25). Symbiont density (symbionts μm^{-3}), symbiont population size, and host cell volume data were fit to GAMs (as previously described), where temperature was the explanatory variable.

- *Growth rates and bacterivory measurements*

Three replicate 40 ml cultures of each cell type (i.e. intact *P. bursaria* and isolated symbionts) were established separately at each assay temperature (15, 20, 25, 30 and 35°C) under a 12:12 h light/dark cycle (~100 µmol PAR m-2 s -1) under standard culture conditions. Cultures were incubated for 4 days and sub-samples were fixed in 3% glutaraldehyde and 0.3% formaldehyde at the start and end of the incubation period. Abundance/density was enumerated via imageJ analysis (Schindelin *et al*. 2012) of fluorescence microscopy images for hosts or via flow cytometry for symbionts. Growth rate was calculated using the decadic logarithm of cell counts and assuming exponential growth using the 'lmList' function in the 'lme4' R package (Bates *et al*. 2015). Growth rates were then fit to a GAM, as previously described, where temperature was the explanatory variable.

For host bacterivory estimates, the *P. bursaria* cultures described above were plated on Luria-Bertani (LB) agar – a typical nutrient-replete medium commonly used for the enumeration of bacteria – and the number of colony-forming units (CFUs) per mL were recorded after 0 and 7 days. The number of bacteria consumed per host at each temperature was then estimated by calculating the reduction in CFUs mL^{-1} across the one-week period. Non-treatment controls were used to adjust for bacterial growth. The 'control' mean change in CFUs mL^{-1}, calculated using 3 replicate control treatments at each temperature, was subtracted from treatment estimates. CFUs consumed mL^{-1} were then divided by mean *P. bursaria* cell counts mL^{-1} and by mean *P. bursaria* cell volume at each temperature to control for differences in bacterivory

as a result of population and cell size, and expressed per day. These data were then fit to a GAM, as previously described, where temperature was the explanatory variable.

Results

Metabolic thermal responses

To examine the metabolic impacts of temperature change on our microbial photo-symbiosis, we exposed symbiotic *P. bursaria* to a range of temperatures between 13°C and 43°C and quantified its resultant rates of GPP and R. These fluxes followed unimodal responses, where rates increased with temperature up to optima and rapidly declined with further warming. The gradients of these responses measured before reaching the optima (E_a), which represent the sensitivities of the processes to temperature (see Padfield et al. 2016), were different: a higher E_a was associated with R and thus R was more thermally sensitive than GPP (Figure 1a; Table 1). Due to this difference in the temperature-sensitivities of these key metabolic fluxes, the fraction of autotrophic carbon available for growth (CUE; 1-R/GPP) declined with temperature across the gradient ($F=38.34$, $edf=3.48$, $P<0.000001$; Figure 1b).

- *Growth*

Holobiont (see Introduction; Box 3) growth rate varied with temperature ($F = 29.8$, $edf = 3.555$, $p<0.0001$; Figure 2a). It appeared to increase with warming between 15°C and 20°C and then remain relatively constant between 20°C and 30°C, before declining with further heating (Figure 2a). Free-living symbiont growth also varied with temperature, broadly following a unimodal response ($F=55.25$, $edf=2.867$, $P<0.00001$; Figure 2b).

- *Symbiont density*

Symbiont population size within hosts varied with temperature ($F=11.48$, $edf=2.382$, $P<0.001$; Figure 2c) and showed a broadly similar response to symbiont growth rate (Figure 2b; 2c), whereby both growth rate and symbiont population size increased with temperature to a maximum and then declined with further heating in a unimodal fashion.

Host cell volume varied with temperature ($F=66.21$, $edf=3.912$, $p<0.0001$; Figure 2d). Symbiont population size expressed per unit volume of host cell (i.e. symbiont density) to control for this host cell volume variation also varied with temperature ($F=32.07$, $edf = 3.828$, $p<0.0001$). Symbiont density was lowest at low temperature,

increasing approximately two-fold to a maximum of 1.6e-04 ± 5.8e-06 symbionts μm^{-3} at 27.0°C and declining rapidly at greater temperatures (Figure 2e).

- *Bacterivory*

We investigated the degree to which bacterivory varied in response to temperature by estimating the number of bacterial cells consumed per host across the temperature gradient within a one-week incubation period (using the appropriate controls to account for changes in bacteria counts not due to consumption; i.e. growth). The numbers of colony-forming units (CFU) consumed per *P. bursaria* cell per day (corrected for *P. bursaria* cell volume to account for differences in grazing associated simply with cells of different sizes) varied with temperature ($F = 67.71$, $edf = 3.906$, $p<0.0001$; Figure 2f); we observed an approximately tenfold decline with temperature, from a consumption of 0.0620 ± .00387 CFU μm^{-3} day^{-1} at 15°C to a minimum of 0.00797± 0.00352 at 28.9°C. Notably, bacterivory then increased at the highest temperatures; bacterivory reached a maximum of 0.0818 ± 0.00387 CFU μm^{-3} day^{-1} at 35°C.

Discussion

We hypothesised that the differential temperature-sensitivities of R and GPP (Lopez-Urrutia *et al*. 2006; Anderson-Teixeira *et al*. 2011; Yvon-Durocher *et al*. 2010, 2012) would reduce the capacity of symbionts to engage in metabolite trade with their

hosts and reduce their growth capacities due to reductions in autotrophic CUE (Padfield *et al*. 2016). This process has the potential to directly constrain symbiont abundances within hosts at high temperature, representing one simple explanation for the apparent sensitivity of photo-symbioses to breakdown in response to warming in nature. In broad agreement with this idea, we found that hosts harboured fewer symbionts and consumed more bacteria with both warming and cooling relative to symbiont growth optimum temperature, suggesting that the thermal responses of the photo-symbiosis depended simply on the thermal niche of symbionts.

In agreement with past work (Padfield *et al*. 2016), the fraction of photosynthetically-fixed carbon available for growth decreased with warming, since R was more temperature-sensitive than GPP (Lopez-Urrutia *et al*. 2006; Anderson-Teixeira *et al*. 2011; Yvon-Durocher *et al*. 2010, 2012). If hosts had sufficient control over their symbiont loads in response to temperature change (for potential mechanisms see Kodama & Fujishima, 2008; Kadono et al. 2004; Lowe et al. 2016; He et al. 2019), they might regulate symbiont load as a result of this constraint (e.g. they could down-regulate the abundance of symbionts; see Chapter 1; Box 2). However, symbiont load increased and then declined across the thermal gradient in a unimodal fashion, and this response broadly followed the growth responses of isolated symbionts. This clear link between symbiont growth rate and symbiont abundance within hosts suggests that the latter may be a simple function of the former, and that effects based on growth dynamics were probably dominant over any ‘active’ host regulation mechanisms, in agreement with previous work (Iwai, Fujiwara & Tamura, 2016). Notably, this response means that departures from the symbiont thermal growth optimum (~25°C), by warming or cooling, leads to losses of symbionts within hosts.

The rate of bacterivory followed the inverse of the symbiont load response across the temperature gradient, suggesting that grazing is potentially a simple function of symbiont density and thus an indirect result of symbiont growth rate. It is unclear whether grazing rate is under active host control, or underpinned by physical constraints associated with housing symbionts. For example, a large symbiont population size potentially inhibits the intracellular processing of food vacuoles, since both activities clearly occupy space within the cytoplasm. Furthermore, symbionts are housed in derivatives of the host's digestive vacuoles (reviewed in Fujishima, 2009); it is thus possible that housing such a population limits the supply of membrane constituents required for phagotrophy (and thus bacterivory). Regardless of the mechanisms, these findings suggest that hosts are constrained to graze secondarily as a response to changes in the sizes of their resident symbiont populations.

Broadly, symbiont load was highest and bacterivory lowest where symbiont growth rate was highest (i.e. warming or cooling relative to the optimum for symbiont growth, which occurred at ~25°C, coincided with declines in symbiont load and increases in bacterivory). These responses suggest that the *P. bursaria-Chlorella* spp. association became increasingly relatively heterotrophic with both warming and cooling relative to the optimum temperature for symbiont growth. Strikingly, this suggests that these ecological responses are primarily driven by symbiont growth dynamics, and that such dynamics are likely dominant over the tendency for warming per se to induce increased heterotrophy: previous work on a different form

of mixotroph (i.e. chloroplast-bearing protists capable of heterotrophic grazing) demonstrated increased grazing rates with warming (Wilken *et al*. 2013), and the authors reconciled their findings with general observations that the rates of heterotrophic processes tend to increase more rapidly with temperature than photosynthetic autotrophy (e.g. Rose & Caron, 2007; Yvon-Durocher *et al*. 2010; and see Wilken *et al*. 2018 and references therein). While our photo-symbiotic ciliate indeed fit this pattern with warming (relative to optimum temperature), the reason was likely in fact due to symbiont growth responses, and not metabolism per se. Thus, in agreement with a recent study (Iwai, Fujiwara & Tamura, 2016), growth dynamics are important determinants of ecological characteristics (i.e. symbiont load) in photo-symbioses; here, we extend this idea to show that such dynamics are temperature-dependent and influence both symbiont load and bacterivory (and thus the likely relative heterotrophy/autotrophy carried out by the association).

Conclusion

In conclusion, our study shows that algal thermal growth responses are likely to be critical in determining the responses of photo-symbioses to contemporary warming. The observed relative reductions in symbiont abundance and increased bacterivory in our photo-symbiotic ciliate, likely caused by thermal disruption of symbiont growth, carry clear implications in the context of natural communities; here, microbial photo-symbioses are key sources of production, bacterivory and species abundance (Berninger *et al*. 1992; Unrein *et al*. 2007; Zubkov & Tarran, 2008; Hartmann *et al*. 2012; Sonntag *et al*. 2011; Summerer *et al*. 2008; Sanders, 1991, 2011; Decelle, Colin & Foster, 2015; Baldauf, 2008). Thus, the relative balance between autotrophy

and heterotrophy in such associations – and when such shifts occur – is likely to be important in assessing the risk of ecological consequences in response to contemporary climate change.

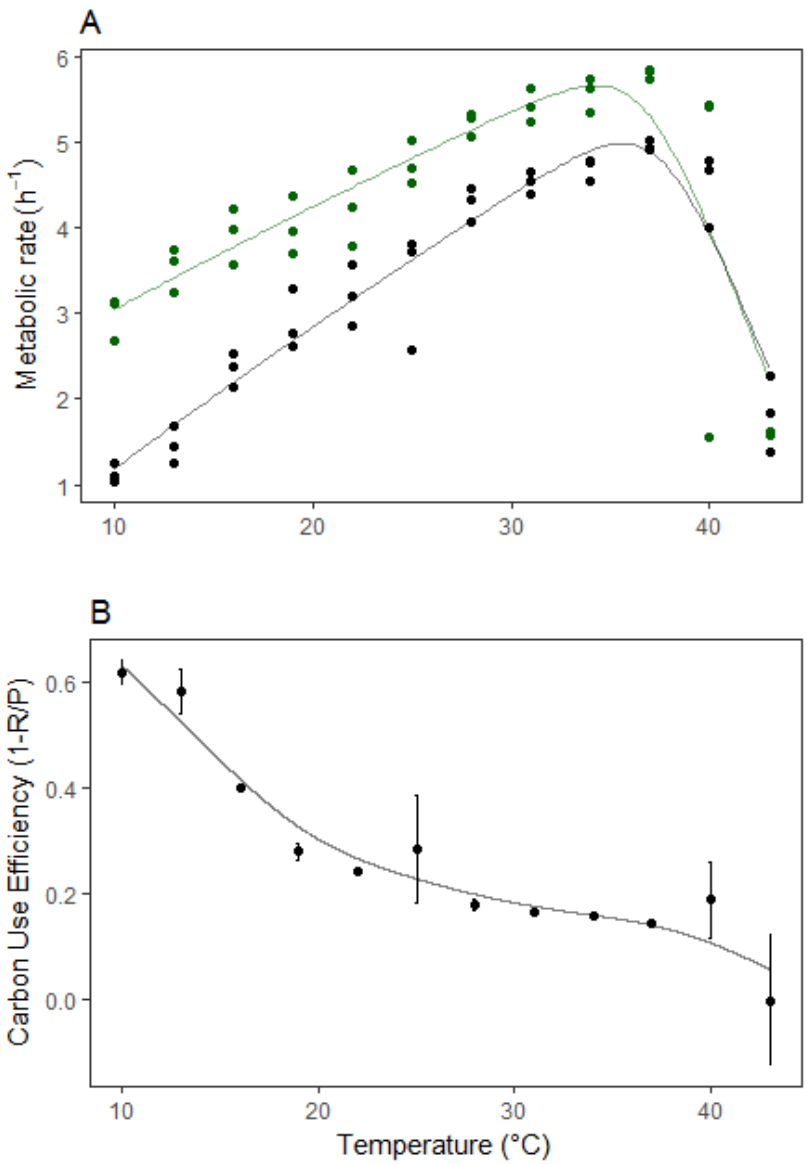
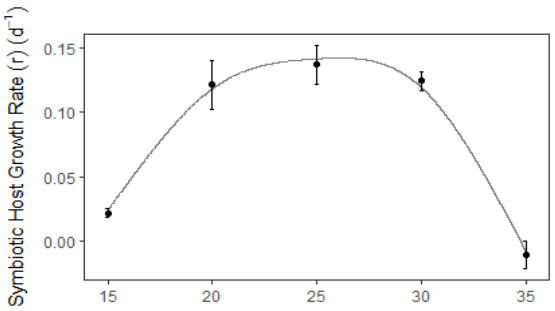


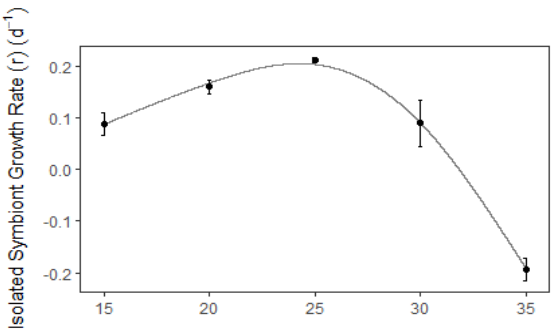
Figure 1: Metabolic flux and carbon-use efficiency across a temperature gradient

(a) Metabolic flux (O_2 h^{-1}). Green lines/points indicate gross primary production, black lines/points represent respiration. Lines represent fitted modified Sharpe-Schoolfield equations (see the statistical procedures outlined in *Methods*). (b) Theoretical carbon-use efficiency (1-R/GPP). Points represent means ±SE.

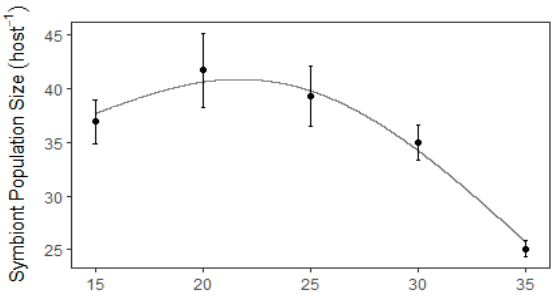
A


Symbiotic Host Growth Rate (r) (d^{-1})
0.15
0.10
0.05
0.00
15
20
25
30
35

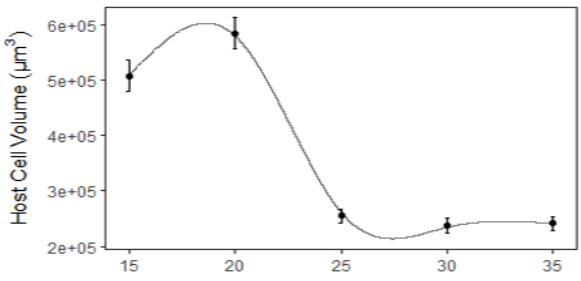
B


Isolated Symbiont Growth Rate (r) (d^{-1})
0.2
0.1
0.0
-0.1
-0.2
15
20
25
30
35

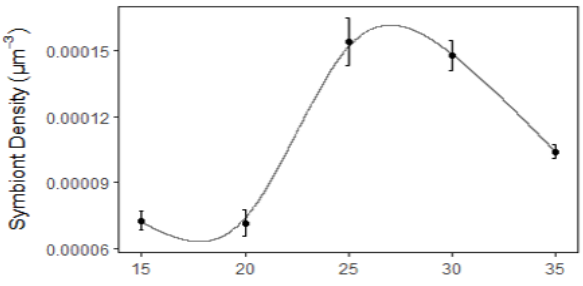
C


Symbiont Population Size (host^{-1})
45
40
35
30
25
15
20
25
30
35

D


Host Cell Volume (µm^3)
6e+05
5e+05
4e+05
3e+05
2e+05
15
20
25
30
35

E


Symbiont Density (µm^{-3})
0.00015
0.00012
0.00009
0.00006
15
20
25
30
35

F

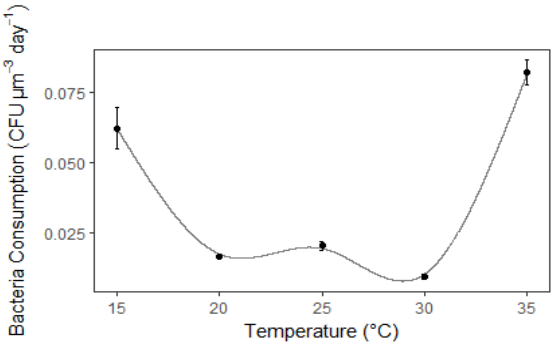

Bacteria Consumption (CFU µm^{-3} day^{-1})
0.075
0.050
0.025
15
20
25
30
35
Temperature (°C)

Figure 2: Temperature-responses of various traits

(3 replicates at each temperature for each trait). Points represent means ±SE. Displayed lines represent fitted GAMs to the data. (a) Growth rate of symbiotic hosts (r, d^{-1}). (b) Growth rate of isolated symbionts (r, d^{-1}). (c) Symbiont population size within hosts. (d) Host cell volume (μm^3). (e) Host symbiont density (symbionts per μm^3 host). (f) host bacterivory (colony-forming units consumed per μm^3 host per day).

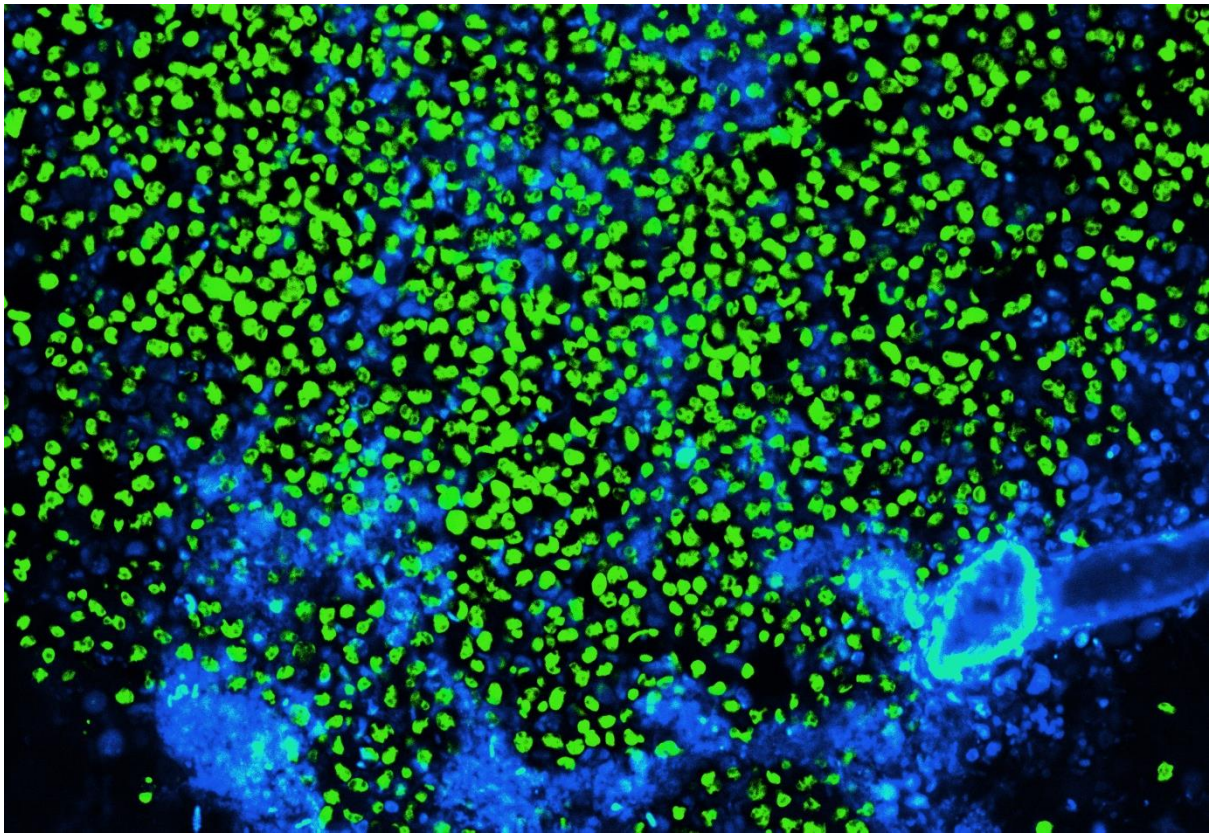
flux	estimate (eV)	std. error	test statistic	p. value
R	1.183497	0.07865236	15.04718	4.55127E-16
GPP	0.8555996	0.1323035	6.466945	3.27335E-07

Table 1: Activation energies of holobiont respiration and photosynthesis

Activation energies of holobiont (i.e intact host and symbionts) respiration and gross primary production (eV). These parameters were estimated by fitting modified Sharpe-Schoolfield equations to the appropriate metabolic flux temperature response curve and are commonly used to compare the temperature-sensitivities of metabolic rates; i.e. higher estimates indicate greater temperature-sensitivity (see Methods).

Chapter 4

The divergent evolutionary responses of a widespread photo-symbiosis to long-term warming



“Evolutionary biology is the key to predicting how the world will change”

- Bell & Collins, 2008

Abstract

Photo-symbioses are important ecological interactions, underpinning both significant biodiversity and critical ecosystem services. Much research has focussed on the responses of these associations to contemporary climate change, with the impacts of warming remaining a research priority. However, the majority of this work has focussed on short-term responses, and comparably little is known about the long term evolutionary responses of photo-symbioses global warming. Here, we use ~10.5 (~21 generations) of controlled temperature change to investigate the evolutionary responses of a microbial photo-symbiosis – the Paramecium bursaria-Chlorella spp. association – to long-term experimental warming (+5°C). Following these temperature regimes, we found that there was an increase in the thermal optima for growth with no change in maximum growth rate. Concurrently, symbionts isolated from the long-term warming treatment were able to grow on inorganic nitrogen sources, suggesting that they had gained/re-gained the capacity for free-living growth. Thus, we found that warming produced an adaptive growth response in holobionts but also appeared to drive the evolution of increased autonomy in symbionts, suggesting that two disparate evolutionary trajectories can be simultaneously followed by photo-symbioses. In the context of the ecological role of photo-symbioses, we found that warm-adapted holobionts had significantly lower rates of primary production (strikingly, net primary production (NPP) rates approached ~0), suggesting that the ecological function of photo-symbioses can change substantially with warming.

Introduction

Symbiotic mutualisms are important ecological interactions. Historically, such interactions gave rise to the eukaryotes (e.g. Lane, 2014; Frank, 1995); earlier, the first genomes – sometime near the origin of life – formed via symbiotic associations between separate replicators (Frank, 1995); a symbiosis between fungi and the roots of land plants drove plants to invade the terrestrial environment (Kiers *et al*. 2010). In extant ecology, all organisms depend upon mutualisms; key associations include plant-pollinator relationships (upon which over half of all extant flowering plants depend) (Landry, 2010); gut microbiota, which are essential for survival of hosts (e.g. Li *et al*. 2008); and the associations between chemosynthetic bacteria and marine Annelids (enabling non-photosynthetic primary production in deep-sea ecosystems; e.g. Smith 2012). Contemporary climate change has already degraded many important mutualisms (Kiers *et al*. 2010) and global mean surface temperature is predicted to rise by an increment of up to 4.8°C by 2100 (IPCC, 2014), risking the further degradation of these associations. Accordingly, understanding the likely future impacts of such temperature-driven changes on mutualisms and their wider ecosystems represents an ongoing major challenge for ecologists.

An important class of symbiotic mutualism sensitive to warming is photo-symbiotic mutualisms (Lesser, 2011), being responsible for ~50% of all marine photosynthesis (and thus a significant portion of the total global carbon budget; Baldauf, 2008), and much of both freshwater and marine biodiversity (Bailly *et al*, 2014; Davy *et al*, 2012; Weis, 2010; Sonntag *et al*, 2011; Summerer *et al*, 2008). Predictions about the future state of photo-symbioses and important ecosystem services that rely upon them

such as coral reefs are highly dependent on the capacity of photo-symbioses to adapt in the face of warming. However, many ecologically-important symbioses including the coral-symbiont association feature hosts which are slow growing and difficult to cultivate (Chakravarti & van Oppen, 2018), and as such evolutionary responses have been difficult to study directly; yet evolutionary studies are now essential in order to better understand the likely trajectories of symbioses in a warmer world (Baker et al. 2008; Kiers et al. 2010). Notably, while the thermal responses of autonomous symbionts of corals have been cultivated and studied over multiple generations – research which has revealed that autonomous symbionts of corals can rapidly adapt to warming (Chakravarti & van Oppen, 2018), paralleling the conclusions of other studies on free-living phytoplankton (Padfield et al. 2016; Schaum et al. 2017) – no studies involving experimental evolution have investigated the responses of any photo-holobiont to warming (as far as we are aware). This is despite the fact that such studies examining thermal adaptation under monitored conditions have been conducted in other symbioses such as squid-bacteria associations (e.g. Cohen et al. 2019), and more broadly, a wide range of studies have observed the responses of symbioses to various environmental stressors and/or experimental conditions. For example, recent research has shown that environmental stress could cause the extinction of fig pollinators (Harrison, 2000; Jevanandam, Goh & Corlett, 2013), bacterial symbionts of stink bugs, beetles and other insects can be lost as a result of warming (Wernegreen, 2012; Kashkouli, Fathipour & Mehrabadi, 2019; Kikuchi et al 2016; Prado et al. 2010; Six & Bentz, 2007; Dysthe, Bracewell & Six, 2015), defensive symbioses can break down in response to warming, for example in aphid-bacteria and ant-plant mutualisms (Doremus et al. 2018; Mooney et al. 2019; Fitzpatrick et al. 2014), the pathogenicity

of *Wolbachia* symbioses can be increased with warming (Rohrscheib et al. 2016), nitrogen addition (e.g. through fertilisers) favours less cooperative mutualists in the legume–rhizobium mutualism (Weese et al. 2015), the timing of pollinator mutualisms can be disrupted due to changes in climate (Robbirt et al. 2014; Warren & Bradford, 2014; Warren, Bahn & Bradford, 2011), extreme heat events can break down temperate pollinators (Sutton et al. 2018) and tree-scatterhoarder mutualisms can transition towards antagonism in response to environmental stress (Sawaya et al. 2018). Studies have also observed the transitioning of mutualisms 'in the other direction' (i.e. from parasitism to mutualism). For example, recent research has shown that *Wolbachia* can evolve to become mutualistic in natural populations of *Drosophila* (Weeks et al 2007; note that this study followed *Drosophila* populations for 20 years), mutualism can evolve in experimental pathogenic virus populations (Shapiro and Turner, 2018), a fungal pathogen can evolve into a mammalian gut symbiont (Tso et al. 2018), a defensive symbiosis can rapidly evolve from a parasitic interaction (King et al. 2016), a plant pathogen can evolve into a legume symbiont (Marchetti et al. 2010; Su Hua Guan et al. 2013) and plant-virus parasitisms can become mutualistic (Hily et al. 2016). Research has also shown that algal-bacterial interactions can transition from antagonism to commensalism (Gonzalez-Olalla et al. 2018) and – perhaps most strikingly – a range of studies have documented the establishment of novel mutualisms from previously non-interacting partners. For example, novel symbioses have been established between yeast and algae (Naidoo et al. 2019), bacteria and archaea (Hillesland & Stahl, 2010), fungi and algae (Hom & Murray, 2014), bacteria and insects (Hosokawa et al. 2016), different bacterial species (Hosoda et al. 2011), bioluminescent bacteria and squid (Schuster, Perry & Cooper, 2010) and ciliates and cyanobacteria (Ohkawa et al. 2011). Despite

phylogenetic evidence suggesting that transitions between mutualism and parasitism have occurred relatively rarely over macroevolutionary timescales (Sachs & Simms, 2006; Sachs, Skophammer & Regus, 2011; Moran & Wernegreen, 2000; Frederickson, 2017), this suite of studies showing that symbioses can transition between mutualism, parasitism and autonomy is arguably reason for concern for the trajectories of mutualisms and their long-term management, such as in corals (Baker et al. 2008), in the face of climate change.

Thus, how photo-symbioses could respond to global warming is uncertain, especially regarding the timescales under which adaptation could occur (e.g. Baker et al. 2008); yet this issue remains at the forefront of environmental concerns (IPCC, 2018; 2019). In this study, we investigate the evolutionary responses of photo-symbiotic partnerships in response to warming through the use of tractable, microbial photo-symbiotic organisms – the ciliated protist *Paramecium bursaria* and its algal symbionts, *Chlorella* spp. (e.g. see Minter *et al*. 2018; Lowe, 2016). Specifically our aim was to examine 1) the evolutionary trajectory for photo-symbioses under warming, 2) the capacity of holobionts to adapt to warming, and 3) the changes in the ecological function of the photosymbiosis (assessed here as grazing rate and NPP) and thus the potential wider ecological consequences of the evolutionary responses of photo-symbioses to warming. To do this, we exposed holobionts to 5°C of warming (approximating the maximum mean temperature increase predicted by the IPCC by 2100; IPCC, 2014) and measured holobiont growth rate (i.e. hosts with resident symbionts), metabolic rates, symbiont load (i.e. the number of symbionts associated with each host), and the free-living growth capacity of symbionts (assessed here as their ability to grow on an inorganic nitrogen source).

Methods

Cultures and long-term temperature treatments

Paramecium bursaria is a widespread and abundant freshwater ciliate (see Minter *et al*. 2018 and references therein) that harbours symbiotic *Chlorella spp*. algae. The particular strain (HA1g, National BioResource Project) used in this study was originally isolated in Hirosaki, Japan in 2010, and had been maintained in our laboratory at 25°C prior to the experiments for ~2 years. *P. bursaria* cells (i.e. symbiotic hosts with their intracellular resident symbionts living inside them) were originally established in six replicate 200mL stock cultures at each of three long-term temperatures: 20°C ('cooled'), 25°C ('ambient') and 30°C ('warmed'). Each culture was sub-cultured intro fresh growth medium every 2 weeks to allow continuous growth, where the growth medium comprised protozoan pellet/Volvic medium (PVM) inoculated with a clonal strain of *Serratia marcescens* for 48 hours prior to use (grown at a single temperature – 25°C – to control for food source 'quality') to provide an abundance of bacterial prey. Cultures were kept under a 12:12 h light/dark cycle (~75 µmol PAR m^{-2} s^{-1}) where they were gently shaken to maintain uniform mixing (~60 RPM). Cultures were transferred and maintained in this manner for 295 days, after which a number of measurements were taken for each experimental replicate (see below).

The temperature regimes were established 'abruptly'; that is, cultures were placed immediately at the different temperatures. While this temperature change is clearly unrealistic in the natural world, it follows the warming regime in other recent studies

(e.g. Zhang et al. 2019) and it was our goal to assess potential evolutionary trajectories rather than understand what will happen in real world ecosystems, which could better be achieved by mesocosm experiments (e.g. Schaum et al. 2017). A 'cooling' treatment was also included to enable an understanding of whether warming per se, as opposed to temperature change, generates the observed results.

Thermal responses: Metabolism

To characterise the metabolic thermal responses after long-term temperature treatments, we measured NPP (via oxygen evolution at different light intensities) and respiration (R) (via oxygen evolution in the dark) in 1mL aliquots of each experimental replicate (see above). Prior to measurements, *P. bursaria* cells were washed on 10µm filters using Volvic to remove bacteria and free-living algae and then acclimatised to the assay temperature (20°C, 25°C and 30°C) for 30 minutes in the dark. Oxygen evolution measurements were conducted following the methods reported in Chapter 2.

Carbon-use efficiency (CUE) and NPP were each fit to linear mixed models using the 'lme' function in the 'lme4' package (Bates *et al*. 2015) in R statistical software (v3.2.0) (R Core Team, 2014), where each was the dependent variable in their respective model. In both models, assay temperature and long-term treatment were the explanatory variables. Random effects were determined at the level of replicates nested within long-term treatment. We also fit CUE and NPP at long-term growth

temperature to general linear models, where the respective trait was the dependent variable and long-term treatment the explanatory variable.

Thermal responses: Holobiont growth

Each replicate was established separately at each of 6 assay temperatures (15, 20, 25, 27.5, 30 and 32.5°C) under a 12:12 h light/dark cycle (~100 µmol PAR m-2 s -1) in 40mL cultures using the same media reported in '*Cultures and long-term temperature treatments'*. Cultures were first incubated for 4 days (to allow for potential acclimation processes to take place). Cultures were then diluted until the starting cell concentration was ~100 cells mL^{-1} and incubated for a further 4 days; samples were fixed in 3% glutaraldehyde and 0.3% formaldehyde at the start and end of this 4-day incubation period, enumerated via manual imageJ analysis (i.e. manual counts conducted on each image) of fluorescence microscopy images at 10x magnification (Leica TCS SP8; Leica Microsystems; Wetzlar, Germany) and growth rate calculated using the decadic logarithm of cell counts (assuming exponential growth using the 'lmList' function in the 'lme4' package (Bates *et al*. 2015) in R statistical software). Cultures were also photographed and cell counts performed after 6 days in the same manner as described.

Following previous studies, growth rates were then fitted to a modified Sharpe-Schoolfield equation for high temperature inactivation (see Padfield *et al*. 2016; Schaum *et al*. 2017; Schoolfield *et al*. 1981) using non-linear least squares regression; fits were determined using the 'nls_multstart' function in the 'nls.multstart'

package (Padfield & Matheson, 2018) in R statistical software. This package compares AIC values to identify the parameter set, drawn from a uniform distribution, which best characterises the data. The goodness of fit values of the selected models were examined graphically and via assessment of pseudo-R2 values. These parameter sets were used to calculate an optimum temperature value for each experimental replicate (i.e. the temperatures where growth rate is highest), which could be compared between long-term temperature treatments. Optimum temperature was calculated using the formula:

Optimum Temperature (in K) = ((Eh*Th)/(Eh + (8.62e-05 *Th*log((Eh/Ea) - 1))))

Where Eh is the value of the deactivation energy (in eV), Th is the temperature at which the substrate is half high-temperature suppressed (in K) and Ea is the activation energy (in eV) (see Padfield & Matheson, 2018; Padfield *et al*. 2016). Optimum temperature values and maximum fitted growth rate estimates from these fitted Sharpe-Schoolfield curves were compared between long-term temperature treatments using linear models.

Thermal responses: Symbiont load

The same replicates established for the growth measurements were used to assess symbiont load. Samples at the 6-day time point (following the 4-day 'acclimation' period) were photographed at 10x using fluorescence microscopy; these images were analysed for mean fluorescence intensity using the 'mean gray value' parameter obtained using the 'Analyze Particles' base function in imageJ. The returned intensity values were then converted into symbiont load values (i.e.

symbiont density within hosts expressed as symbionts μm^{-1}) using the empirically derived formula (see Figure S2):

$$L = (m*I) + c$$

Where L = symbiont load (μm^{-1}), m = 1.25896e^{-05}, c = -5.401277e^{-05} and I = intensity (mean gray value). The constants m and c were empirically derived by lysing reference populations of holobionts of known intensities, directly enumerating symbionts within each host via flow cytometry (see below) and constructing a linear model within which fluorescence intensity was the explanatory variable and symbiont load was the dependent variable.

Direct symbiont density counts within hosts were achieved by sonicating 15 *P. bursaria* stocks kept at a selection of assay temperatures (15, 20, 25, 30 and 35°C) during mid-log growth phase (using 3 pulses of 10s at 90% amplitude over ice; each culture was 10mL) in order to rupture host cell membranes; we enumerated the resultant released symbiotic algal cells using flow cytometry (BD Accuri C6; BD). Flow cytometry involved simply passing these lysed cultures through a flow cytometer where the number of algae detected in a known volume (10µL) of the sample was based on chlorophyll fluorescence while the culture was penetrated by a laser. The system was first calibrated as recommended and detailed by the manufacturer and negative controls (i.e. where growth medium alone was run through the flow cytometer) were first compared with algal cultures in trial runs to confirm that isolated algae in our experiments could be successfully detected and counted. Direct symbiont density counts were controlled for the density of *P. bursaria* hosts by dividing the number of counted algae by the number of hosts (enumerated

via imageJ analysis using the ‘blur’ followed by ‘Find Maxima’ base functions (Schindelin *et al*. 2012); the number of hosts lysed per replicate was typically ~1000) of fluorescence microscopy images at 10x magnification (Leica TCS SP8; Leica Microsystems; Wetzlar, Germany)). Symbiont population sizes per host were also divided by mean cell volume estimates in order to control for changes in host cell volume; measurements of *P. bursaria* cell volume (μm^3) were performed manually on bright field microscopy images using the provided manufacturer software (LAS X; Leica Microsystems) where length and width measurements were taken and volume calculated assuming that cells were prolate spheroids ($Volume = 4/3\pi a^2 c$; where *a* and *c* are the polar radii; the number of hosts measured per replicate was 25).

To allow for a non-linear response, we fitted the resultant symbiont load estimates (converted from cell fluorescence intensities as detailed above) to a generalised additive mixed model (GAM) using the ‘gam’ function in the ‘mgcv’ package (Wood, 2006) in R statistical software, where assay temperature and long-term treatment were explanatory variables and symbiont load was the dependent variable. Random effects on the intercept were determined at the level of replicates nested within long-term treatment. We also fit symbiont load at long-term growth temperature to a general linear model, where the respective trait was the dependent variable and long-term treatment the explanatory variable.

Thermal responses: Bacterivory

The same replicates established for the growth measurements detailed in '*Thermal responses: Holobiont growth*' were used to assess bacterivory. For host bacterivory estimates, the *P. bursaria* cultures were plated on Luria-Bertani (LB) agar – a typical nutrient-replete medium commonly used for the enumeration of bacteria – and the number of colony-forming units (CFUs) per mL were recorded after 0 and 7 days. The number of bacteria consumed per host at each temperature was then estimated by calculating the reduction in CFUs mL^{-1} across the one-week period. Non-treatment controls were used to adjust for bacterial growth. The 'control' mean change in CFUs mL^{-1}, calculated using 3 replicate control treatments at each temperature, was subtracted from treatment estimates. CFUs consumed mL^{-1} were then divided by mean *P. bursaria* cell counts mL^{-1} and by mean *P. bursaria* cell volume at each temperature to control for differences in bacterivory as a result of population and cell size, and expressed per day. To allow for a non-linear response, these data were then fit to a GAM, where temperature was the explanatory variable and CFUs consumed day^{-1} the dependent variable. Random effects on the intercept were determined at the level of replicates nested within long-term treatment.

Autonomous symbiont growth

A 10mL sample from each long-term temperature treatment replicate was mechanically lysed via sonication (as previously described) to release symbiotic algae from hosts. These algae were then established separately in two different growth media: one contained inorganic nitrogen (i.e. nitrate) as the nitrogen source

and the other contained organic nitrogen (i.e. bacto-peptone). Both media also contained a modified bold basal medium (BBM) from which the nitrogen source had been omitted. Algal counts at 0 and 4 days after incubation at the respective long-term temperature were conducted via flow cytometry (as previously described) and growth rate calculated using the decadic logarithm of cell counts (assuming exponential growth). Algal growth on inorganic and organic nitrogen was fit to linear models where growth on the appropriate source was the dependent variable while the long-term temperature treatment was the explanatory variable.

Assessing the number of generations passed during the experiment

The number of generations was calculated following Padfield et al. 2016 using the following formula:

$$g = \frac{\Delta T}{\ln(2)/\mu}$$

where ΔT is the time interval of the transfer (d), ln(2)/μ is the doubling time (d) and μ is the growth rate (d^{-1}).

Results

Long-term cultures

The experiment lasted 295 days (~10.5 months). Approximately 21 generations passed in the warming treatment during this time while ~58 passed at

control/ambient temperature and ~55 at the cooled temperature (Table 1). Notably, 2 replicates became extinct in the warming treatment while all other replicates in the other temperature treatments survived.

Metabolism

We analysed the metabolic thermal responses of holobionts following 295 days of experimental temperature change. Holobiont CUE declined with warming in all long-term treatments (Figure 1a). Analyses using linear mixed effects models revealed a significant effect of long-term temperature on CUE (but note that the potential interaction between long-term temperature and assay temperature was not retained in the model; Table S1). This means that while the CUE thermal response was statistically indistinguishable between long-term temperature treatments, long-term temperature significantly impacted CUE (i.e. the intercept). CUE measured at the long-term growth temperature for each long-term treatment was significantly different ($F=81.84$, $df=2,12$, p-value: $p<0.0001$; Figure 1b; Table S2); Tukey's multiple comparisons of means revealed that growth-temperature CUE was lower in the long-term ambient treatment compared to the long-term cooled treatment and that CUE was lowest in the long-term warming treatment (Table S3). Strikingly, mean growth-temperature CUE under the long-term warming treatment was ~0 (CUE = -0.0472±0.262 (± standard error)) compared to a CUE of 0.779±0.0181 and 0.700±0.0108 under the long-term cooling and ambient treatments, respectively. Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Holobiont NPP increased with warming in the ambient and cooled long-term treatments, but declined with warming in the long-term heated treatments (Figure 1c). Analyses using linear mixed effects models revealed a significant interaction between long-term temperature treatment and assay temperature, meaning that the long-term temperature treatment significantly impacted the thermal response of NPP (Figure 1c; Table S1). NPP measured at the long-term growth temperature for each long-term treatment was significantly different ($F=7.489$, $df=2,13$, $p<0.01$; Figure 1d; Table S2); Tukey's multiple comparisons of means revealed that growth-temperature NPP was statistically indistinguishable between the long-term cooled and ambient treatment, but was lower in the long-term warming treatment (Table S3). Strikingly, mean growth-temperature NPP under the long-term warming treatment was roughly 9-times lower and ~0 (NPP = 0.0130±0.0271 (± standard error)) compared to a NPP of 0.0875±0.00659 and 0.0868±0.0118 under the long-term cooling and ambient treatments, respectively. Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Holobiont growth

To assess holobiont thermal growth responses following their respective long-term temperature regimes, we calculated growth rates and fit the data to Sharpe-Schoolfield curves (Figure 2; see Methods). These fitted curves allowed us to compare optimum temperatures for growth ($T_{opt,}$ °C) and maximum growth rates (gr_{max}, day^{-1} on $\log_{10}$ scale) between long-term temperature treatments. Linear models revealed that T_{opt} significantly varied between long-term temperatures

(F=5.0021, df=2,9, p=0.03461; Table S2), but gr_{max} did not (F=0.8766, df=1,14, p=0.365; Table S2). Tukey's multiple comparisons of means revealed that T_{opt} was significantly higher in the long term warmed treatment compared to the ambient treatment (Figure 2; Table S3). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Symbiont load within hosts

Following the long-term temperature regimes, we also assessed symbiont load (i.e. density of symbionts within hosts). Symbiont load broadly followed a unimodal response to temperature in all long-term temperature treatments (Figure 3a). The patterns were fit to GAMs; analyses revealed a significant effect of long-term temperature treatment on both the shape and intercept of the symbiont load thermal response (Figure 3a; Table S4). Symbiont load measured at the long-term growth temperature was significantly different between long-term temperature treatments (F=35.647, df=2,13, p<0.0001; Figure 3b; Table S2); Tukey's multiple comparisons of means revealed that symbiont load was significantly lower in the long-term warming treatment compared to the long-term ambient and long-term cooling treatments. There was no significant difference between the long-term ambient and cooling temperatures (Table S3). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Symbiont autonomy

Symbiont growth on the inorganic nitrogen source varied significantly with long-term temperature treatment ($F=7.200$, $df=2,13$, $p=0.007856$; Table S2). Tukey's multiple comparisons of means revealed that growth was not significantly different between the cooled and ambient treatments, where growth rate was negative or ~0 (Figure 4a; Table S3). Symbiont growth was significantly higher, and was always positive, under the warmed treatment (Figure 4a; Table S3). Symbiont growth on the organic nitrogen source varied significantly with long-term temperature treatment ($F=32.626$, $df=2,13$ DF, $p<0.0001$; Table S2); Tukey's multiple comparisons of means revealed that growth rate was highest at the long-term ambient temperature, lower under the cooled treatment and lowest under the warmed treatment (Figure 4b; Table S3). Notably, growth rate was always negative in the case of the long-term warming treatment but always positive for the other treatments (Figure 4b). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Holobiont bacterivory

Following the long-term temperature regimes, we also assessed holobiont bacterivory (i.e. the rate of prey consumption by holobionts). Bacterivory rate followed a pattern whereby it broadly declined and then increased across the temperature gradient in the long-term cooling and ambient treatments, but it remained comparatively low across the gradient in the long-term warming treatment (Figure 5). The patterns were fit to GAMs; analyses revealed a significant effect of

long-term temperature treatment on both the shape and intercept of the bacterivory rate thermal response (Figure 5; Table S4). Bacterivory rate measured at the long-term growth temperature was significantly different between long-term temperature treatments ($F=695.04$, $df=2,13$, $p<0.0001$; Figure 5; Table S2); Tukey's multiple comparisons of means revealed that bacterivory rate was significantly different between all treatments, where it was highest in the long-term ambient temperature treatment, lower in the warming treatment and lowest in the cooling treatment (Table S3). Tukey's post-hoc testing was conducted using the TukeyHSD base function in R statistical software, which controls for multiple comparisons and unbalanced designs.

Discussion

In this study, we examined the impacts of long-term warming on photo-symbiosis through the use of ~10.5 months of experimental temperature change on a common microbial photo-symbiotic association. Our temperature increment – 5°C – approximates the maximum value of mean temperature increase predicted by the IPCC (IPCC, 2014). Over this time period, which corresponded to ~21 generations, we found that there was an increase in the thermal optima for growth but no increase in maximum growth rate. Concurrently, symbionts isolated from the long-term warming treatment were able to grow on inorganic nitrogen sources, suggesting that they had gained/re-gained the capacity for free-living growth.

The experiment lasted 295 days (~10.5 months), although only approximately 21 generations passed in the warming treatment during this time. This reinforces the

view that the slow growth rate of certain photo-symbioses in the face of environmental stress may be cause for concern in the context of climate change and may hinder research into the evolutionary responses of such associations (Baker et al. 2008; Chakravarti & van Oppen, 2018). On the other hand, our experiment tested just over the upper temperature range predicted by the IPCC for global increase (+5°C used in the experiment; 4.8°C maximum value predicted by IPCC; IPCC, 2014), so this represents the 'worst case' warming scenario and potentially a strong selection pressure. However, there are caveats associated with measuring the number of generations; 1) assessing the number of generations under stressful conditions may underestimate the actual number of generations passed, since it does not take into account the rate of death (i.e. a high population turnover with a high rate of death would yield a low growth rate and thus a low number of generations passed) and 2) although symbiont and host reproduction is synchronised in the *P. bursaria-Chlorella spp*. system (Takashi, 2016), symbionts could potentially escape from hosts (e.g. through host lysis) and grow independently, thus passing through more generations than was measured for holobionts. Hosts have been shown to take up symbionts in culture, potentially enabling such autonomous symbionts access back into symbiosis (Takahashi, 2016 and references therein). In this way, symbiont evolution might be expected to be more rapid than that of hosts, as is suspected to be the case for coral symbioses (Chakravarti & van Oppen, 2018).

Due to the higher thermal sensitivity of respiration compared to photosynthesis (e.g. López-Urrutia et al. 2006; Anderson-Teixeira et al. 2011; Yvon-Durocher et al. 2010, 2012), warming tends to reduce the relative carbon fraction available after accounting for respiratory losses; this balance is referred to as 'carbon-use efficiency (CUE)' (Padfield et al. 2016). In the context of photo-symbiosis, a reduction in CUE is likely to increase the cost of photosynthate transfer for symbionts and decrease the benefit of harbouring symbionts for hosts (because symbionts will likely have relatively less of the produced photosynthate available for trade, driving up its 'price' (Sørensen et al. 2016)). We thus predicted that a fundamental way in which the cost:benefit balance could be shifted, potentially risking the progression towards breakdown, in photo-symbiosis could be via metabolic responses to temperature, due to changes in photosynthetic CUE. We recorded the expected CUE thermal response in all long-term treatments: CUE declined with assay temperature. However, CUE measured at long-term growth temperature was significantly lower in the long-term warming treatment; strikingly, mean CUE was ~0 at growth temperature for warm-adapted holobionts. This suggests that the capacity for metabolite exchange under long-term warming is likely to be disrupted (since there will be a reduced supply of photosynthates available for translocation from symbionts to hosts), potentially reducing the benefit associated with photo-symbiosis for hosts and symbionts. Notably, this metabolic response contrasts with previous laboratory experiments on free-living algae, which showed that phytoplankton were able to metabolically adapt to long-term warming in order to restore thermally-constrained CUE (Padfield *et al*. 2016; Schaum *et al.* 2017). The photo-symbiosis did not appear

to compensate for this metabolic challenge over the ~10.5 month timescale studied here. In fact, CUE was significantly lower under long-term warming compared to the short-term response in long-term ambient holobionts: this implies that long-term warming amplified, rather than compensated for, the loss in CUE.

Such disruption of metabolite trade with long-term warming could generate a selective pressure for hosts and symbionts to turn to alternative means of acquiring the same volume of metabolites that were previously provided via the symbiosis. For symbionts, this could mean acquiring organic nitrogen through the assimilation of inorganic nitrogen found in the environment through the nitrogen assimilation pathway, as is typical of free-living *Chlorella* species (Sanz-Luque et al. 2015). This could further be selected for if hosts expel 'costly' symbionts in response to reduced CUE; *P. bursaria* hosts are thought to control their symbiont densities through mechanisms such as digestion of symbionts (Kodama & Fujishima, 2008), cell cycle regulation (Kadono *et al.* 2004) and potentially nitrogen restriction (Lowe *et al*. 2016; He et al. 2019), and can be observed living autonomously (i.e. free of symbionts) in the wild (e.g. Tonooka & Watanabe, 2002). In agreement with this idea, we found that symbionts liberated from inside holobionts from the long-term warming treatment were able to grow on inorganic nitrogen (where as symbionts from the ambient and cold-treated holobionts grew only on organic nitrogen). Intriguingly, this suggests that symbiont assimilation capacity 'switched' from organic to inorganic nitrogen, likely reflecting an evolved preference for nitrogen sources (i.e. nitrates) that are not thought to be provided by hosts in symbiosis (Albers *et al.* 1982) and thus a transition towards increased autonomy. This idea fits with our observation that more free-living symbionts were observed co-existing with holobionts in warm-adapted

cultures (Figure 4c; although note that these data have not been tested for significance); while it would appear that symbionts become liberated from hosts (e.g. through host lysis or host regulatory mechanisms) at all temperatures, it is likely that the warm-adapted symbionts were able to reproduce outside of the host (utilising inorganic nitrogen contained in the growth medium) and grow into relatively large autonomous populations.

The symbionts at the ambient and cooled temperatures retained the ability to grow on organic nitrogen and did not grow effectively on nitrate. This is as expected, since two independent nitrate reductase gene degradation events have occurred in closely-related *Chlorella* symbiont strains in *P. bursaria* symbioses: one in the nitrate reductase gene and another in a regulatory gene associated with nitrate metabolism (Kato & Imamura, 2009; Kamako et al. 2005). These mutations have incurred the loss in function of the nitrate assimilation pathway (see Sanz-Luque et al. 2015) responsible for enabling the use of nitrates in metabolism. What could be the mechanism of the change in symbiont nitrogen metabolism that has occurred in response to warming in the current study? Selection acting on phenotypic changes caused by de novo mutations or selection of existing phenotypes ("symbiont shuffling"; see Jones et al. 2008) could explain the rapid evolution of the capacity for growth on nitrate. However, it seems unlikely that symbionts could "re-evolve" the nitrate reductase gene or other genes important for nitrate assimilation if they had been previously lost to mutations such as those described above – rather, it is presumably more likely that the pathway was silenced in a reversible manner in the ancestral *Chlorella* symbionts used in the current study. Warming could have acted to remove this silencing effect. This hypothesis could be tested in future work by

transplanting the warm-adapted symbionts (which have “re-gained” nitrogen assimilation) into the cold and ambient conditions to examine whether the nitrogen assimilation capacity can be “turned off again” within one, or a small number of, generations. Furthermore, the genes that have been affected in other strains in past work (Kato & Imamura, 2009; Kamako et al. 2005) could be assessed in the strain used in the current study by comparing genome sequences; this would reveal whether mutations have degraded the genes within the nitrate assimilation pathway or whether a functional/intact system is likely to have instead been “turned off”. It is more challenging to explain why the warm-adapted symbionts, in addition to “re-gaining” nitrate assimilation, also appeared unable to utilise organic nitrogen. This suggests that in addition to a re-activation of nitrate assimilation, there has also been a silencing or mutation at a later stage in the nitrogen assimilation pathway (see Sanz-Luque et al. 2015). Intriguingly, this could have occurred as an adaptive symbiont response to host sanctions, as discussed above. Organic nitrogen provision is thought to be a key way in which hosts regulate the abundance of *Chlorella* symbionts (Lowe *et al*. 2016; He et al. 2019). Since CUE and NPP are low in the warming treatment, host might be expected to impose tighter sanctions on their symbionts as a result of the eroded photosynthetic benefit and photosynthate provision (see see Foster et al. 2017; Sachs et al. 2004; Edwards, 2009); theoretically, this could impose a strong selection pressure on symbionts to “escape” from this host control and become insensitive to organic nitrogen provisioning.

Despite the metabolic responses (i.e. the low CUE and NPP observed under the long-term warming treatment), we showed that holobionts appeared to adapt to long-term warming (evidenced through a shift in thermal growth optima towards the long-

term temperature), where the maximum growth rates achieved under each treatment were not statistically distinguishable. Why did hosts continue to harbour symbionts in spite of low CUE, and what mechanisms enabled this maintenance and adaptation of holobiont growth rate? There are a number of possible answers: 1) The cost:benefit balance of the mutualism for the host was stabilised/restored by other benefits conferred by symbionts that became more important with warming (e.g. photo-protection; Summerer *et al.* 2009). 2) Hosts became more efficient at extracting and/or utilising available photosynthates from symbionts with long-term warming. 3) Hosts increased heterotrophic efforts to compensate for lost carbon. 4) The evolved capacity for symbiont growth on inorganic nitrogen reduced the capacity for control by hosts. While we cannot rule out options 1) and 2) here, heterotrophy did not appear to explain the maintenance of symbiosis, since it was in fact down-regulated in response to long-term warming. However, it is possible that symbionts were able to evade one method of host control due to an evolved incompatibility: *P. bursaria* hosts are thought to provide, and potentially regulate their symbiont populations with (Lowe et al. 2016), organic nitrogen supply and not nitrates while symbionts required nitrates and not organic nitrogen. This could suggest a more parasitic role for symbionts under long-term warming, yet elucidating the fitness impacts of symbionts would require further experiments that include comparisons with autonomous hosts.

As has been shown in our other work (see Chapter 3), the symbiont load response was non-linear, which could be a result of symbiont growth across the temperature gradient (although we did not test this here; See Chapter 3). Intriguingly, there appears to be a trough in the symbiont load thermal response at ~25°C under all three long-term temperature treatments. We note that 25°C is the ancestral

temperature. Thus, one explanation for this could be that hosts exhibit a tighter control over their symbiont abundances because the exchange has had an evolutionary history where it has evolved to become most efficient at ancestral temperature. Past work has shown that hosts likely regulate their symbiont loads in response to abiotic factors in order to gain the maximum benefit from them (Lowe et al. 2016); it is in the host's interest to function with as few efficient symbionts as possible (Lowe et al. 2016). Thus, the troughs could potentially be a result of more effective host control.

Taken together, the observations that symbionts appeared to transition towards autonomy while holobionts simultaneously adapted to high temperature suggest that two divergent evolutionary trajectories were followed by the photo-symbiosis in response to warming: the maintenance of symbiosis (i.e. hosts and symbionts maintain their symbiotic relationship, albeit potentially in a revised form) and the autonomy of one of the partners. Thus, the capacity for two distinct outcomes to occur simultaneously (i.e. mutualism abandonment and maintenance) could represent an important and overlooked outcome for the future trajectories of mutualisms in the face of contemporary climate change; typically, theory seeks to describe one over the other (e.g. Sachs & Simms, 2006). However, whether symbiont autonomy could occur in real-world photo-symbioses in response to warming will depend on a number of factors. Firstly, natural environments could clearly enforce strong selection pressures against symbiont autonomy, for example via the presence of predators, competitors and/or viruses that cause selective mortality in the free-living state. Indeed, viruses are thought to play a role in preventing the occurrence of autonomous *Chlorella* symbionts in the *P. bursaria*

relationship in nature (Hoshina & Imamura, 2009). In addition, nitrogen availability will undoubtedly strongly influence whether symbionts can become autonomous; free-living options are generally more feasible if services previously supplied by symbiosis are readily provided to organisms in the environment (Johnson, 2010). Since our growth medium contained inorganic nitrogen, symbionts likely had access to this alternative nitrogen source (e.g. through expulsion and reabsorption of symbionts by hosts or via host 'leakiness' that enabled nitrates to come into contact with symbiont metabolic machinery) and could thus adaptively 'select' it over host-provided nitrogen compounds. Real-world ecosystems could either enforce or suppress such availability. For example, the use of fertilisers worldwide has risen (Gregory & Ingram, 2000 and references therin); factors that could allow for increased access to nitrates in nature will likely be important, determining the potential for photo-symbiotic organisms to be readily exposed to different nutrient acquirement possibilities. Indeed, such 'nutrient enrichment' is known to have caused degradation in plant–rhizosphere mutualisms (Johnson, 2010). On the other hand, nitrogen-sparse environments are likely to strongly select against autonomous symbionts; indeed, many photo-symbioses are found in oligotrophic environments (Takagi *et al*. 2018 and references therin) where the associations provide clear nutritional mutual benefits.

The potential ecological consequences of thermal adaptation

Since photo-symbioses provide important ecosystem services such as primary production (Baldauf, 2008) and bacterivory alongside other photo-mixotrophs (Berninger *et al*. 1992; Unrein *et al*. 2007; Zubkov & Tarran, 2008; Hartmann *et al*.

2012) in nature, we assessed the potential for these contributions to change with long-term temperature. Holobiont NPP was drastically impacted by long-term warming, but not cooling; strikingly, per capita NPP was close to zero at growth temperature under the long-term warming condition. Given that ~50% of all marine photosynthesis is underpinned by photo-symbiosis (Baldauf, 2008), this could indicate that rates of global photosynthesis are at risk of potentially large reductions in the future as a result of the impacts of global warming on photo-symbioses.

In addition, holobiont bacterivory responses were also significantly impacted by long-term warming. Warm-adapted holobionts displayed comparably invariant low bacterivory rates across a thermal gradient, while the long-term ambient and cooled holobionts rapidly increased bacterivory with temperature. This suggests that in the short term, bacterivory is likely to rapidly increase with warming, but that this response is dampened over evolutionary time. This dampening response could have implications for understanding how food-web dynamics will change with warming. For example, temperature has been shown to increase top-down control within food-webs as a result of grazing (e.g. Gilbert et al. 2016; O'Connor, Gilbert & Brown, 2011; Miller, Matassa & Trussell, 2014; Schaum et al. 2017) and forms part of a mosaic of interacting factors that can impact food webs (Gibert, 2019). However, the photo-symbiosis in the current study appeared to down-regulate grazing in response to long-term warming (compared to a rapid increase that is incurred by short term warming). Thus, increased top-down control could only be transient (i.e. may weaken over evolutionary time). Furthermore, if the growth rates of prey microorganisms increase with temperature in the absence of increasing host predation efforts with temperature, then food webs could become imbalanced in "the other direction".

Indeed, we found that the growth rate of the isolated prey microorganism used in this study (*Serratia marcescens*), which is widespread in natural environments, increased in a linear manner between our experimental temperatures (Figure S1). Thus, understanding the impacts of warming on food-web dynamics will require an understanding of how photo-symbioses as key sources of bacterivory alongside other photo-mixotrophs (Berninger *et al*. 1992; Unrein *et al*. 2007; Zubkov & Tarran, 2008; Hartmann *et al*. 2012) can adapt to warming.

The temperature regimes in this study were established ‘abruptly’; that is, cultures were placed immediately at the different temperatures. While this temperature change is clearly unrealistic in the natural world, it follows the warming regime in other recent studies (e.g. Zhang et al. 2019) and it was our goal to assess potential evolutionary trajectories rather than understand what will happen in real world ecosystems, which could better be achieved by mesocosm experiments (e.g. Schaum et al. 2017). A gradual, or fluctuating, temperature change regime could have produced different outcomes. For instance, the fact that symbionts appeared to evolve increased autonomy was surprising; this could in part be a result of the potentially strong selection pressure imposed by the abrupt and high degree of warming. Regardless, this work proves that evolutionary experiments can and should be conducted in photo-symbioses: the *P. bursaria-Chlorella spp.* association could serve as a useful model for further study. Indeed, although the authors were referring to coral-zooxanthellae associations, the idea of focussing on a few experimentally tractable model associations that could speed understanding of photo-symbioses and their responses to warming has received recent attention (Weis, 2008).

Conclusion

In this study, we examined the impacts of long-term warming on photo-symbiosis through the use of ~10.5 months of experimental temperature change on a common microbial photo-symbiotic association. Our temperature increment – 5°C – approximates the maximum value of mean temperature increase predicted by the IPCC (+4.8°C by 2100; IPCC, 2014). Intriguingly, we found that warming produced an adaptive growth response in holobionts but also appeared to drive the evolution of increased autonomy in symbionts, suggesting that two disparate evolutionary trajectories were followed. Thus, the simultaneous maintenance of symbiosis and reversion to autonomy of one of the partners appears to be a potential outcome for photo-symbioses over evolutionary timescales, and such a dual outcome could be overlooked by contemporary theory (which typically discusses outcomes as discrete phenomena, e.g. Sachs & Simms, 2006). We argued that whether such an outcome could occur in nature will likely depend on a multitude of other factors; inclusion of such factors in future work could reconcile our findings, collapsing the outcome into one or the other. Finally, in the context of the ecological importance of photo-symbioses, we found that warm-adapted holobionts had significantly lower rates of primary production (strikingly, NPP was close to zero at growth temperature in the long-term warming condition); if such responses occur in nature, they would have clear implications for global primary productivity, of which photo-symbioses currently underpin around one half (Baldauf, 2008).

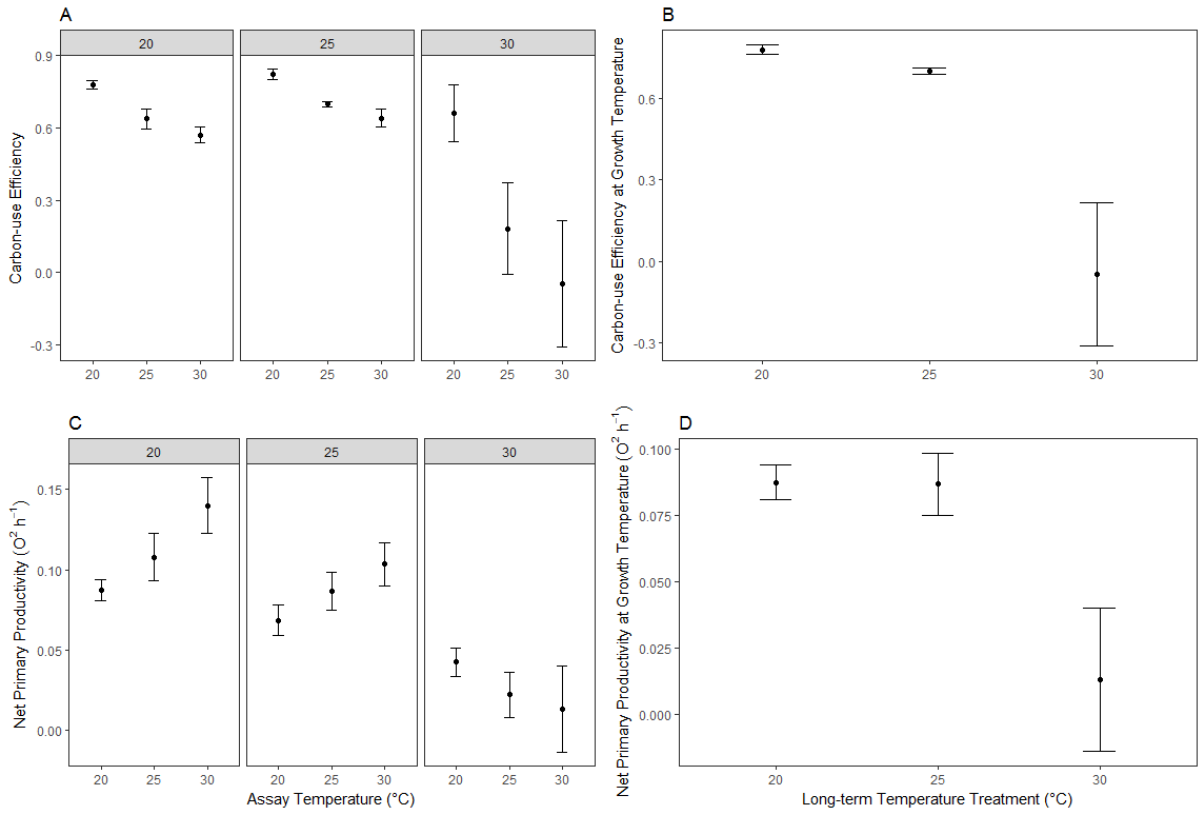


Figure 1: Holobiont metabolic thermal responses

(a) Carbon-use efficiency (CUE) at each assay temperature. Temperature label in grey block indicates the long-term treatment temperature. (b) CUE at long-term growth temperature for each long-term temperature treatment. (c) Net primary productivity (NPP) at each assay temperature. Temperature label in grey block indicates the long-term treatment temperature. (d) NPP at long-term growth temperature for each long-term temperature treatment. Points indicate means with standard errors.

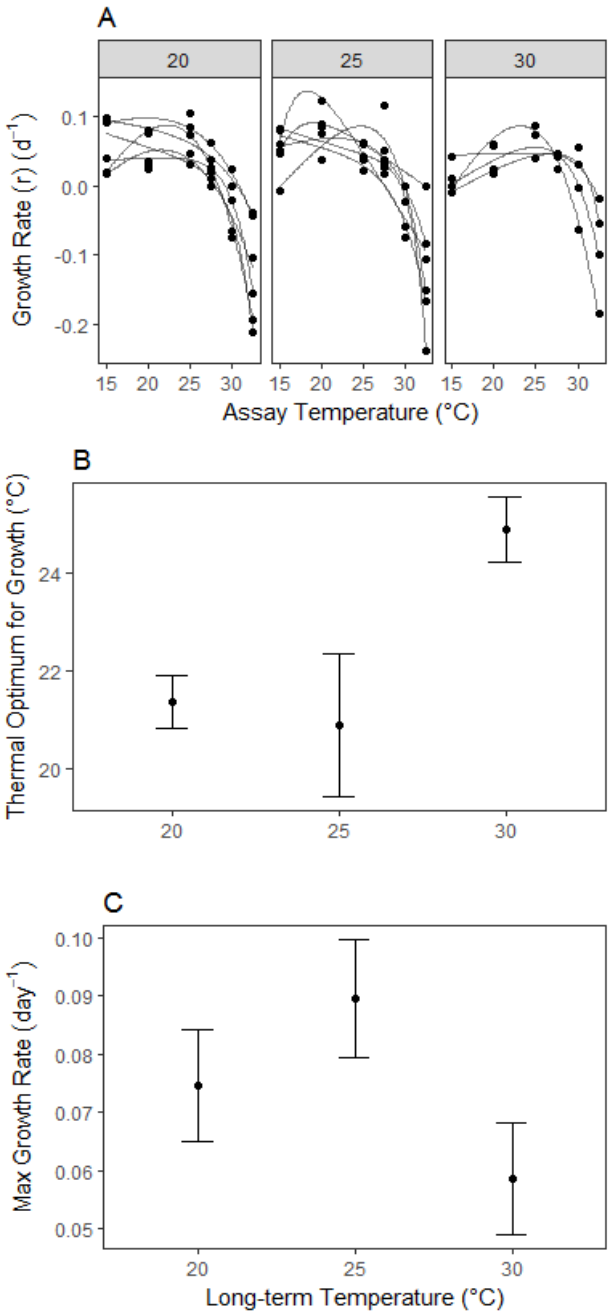


Figure 2: Holobiont growth thermal responses

(a) Holobiont growth rate with fitted Sharpe-Schoolfield curves (see Methods) for each long-term replicate in response to assay temperature. Temperature label in grey block indicates the long-term treatment temperature. (b) Thermal optima for growth for each long-term treatment. These parameters were derived from the fitted Sharpe-Schoolfield equations in (a) (see Methods). (c) Maximum values for growth

rates for each long-term selection temperature. Points indicate means with standard errors.

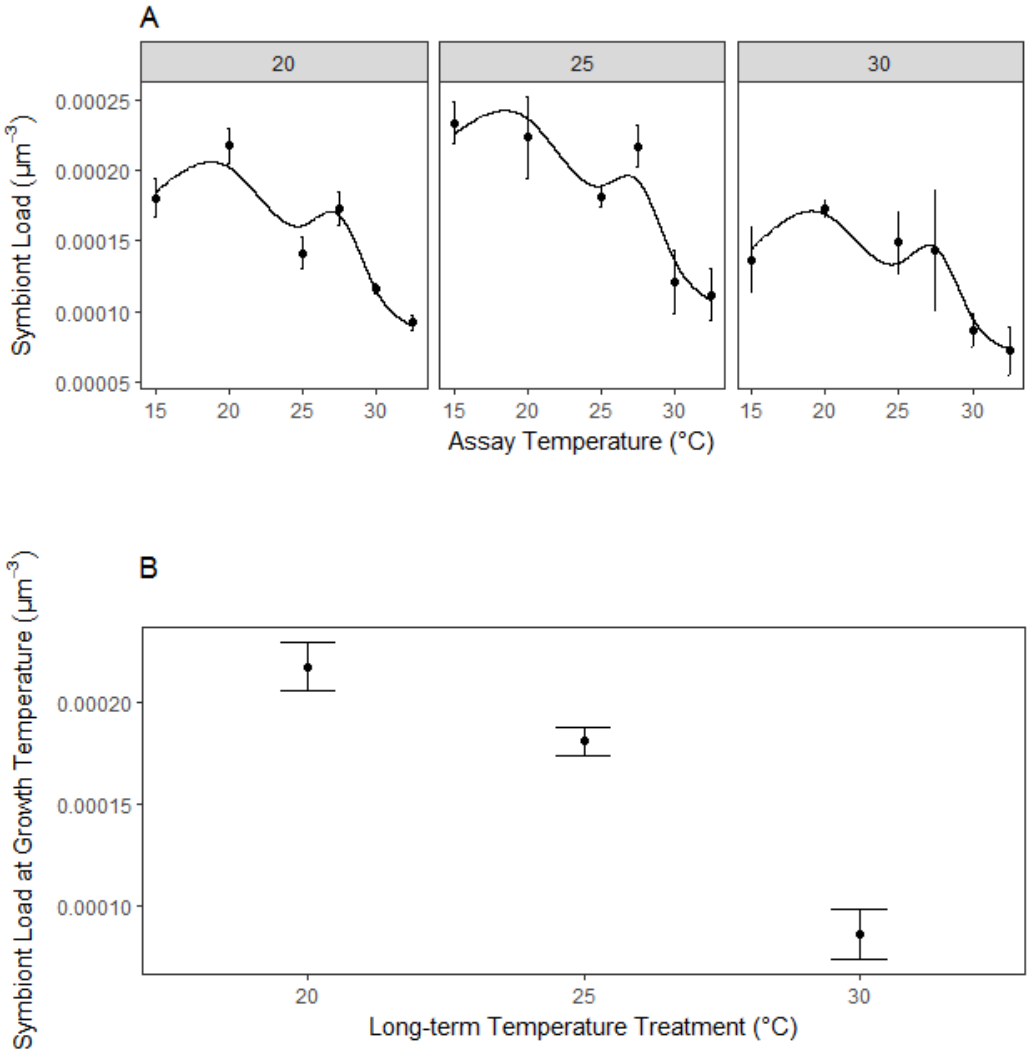


Figure 3: Holobiont symbiont load thermal responses

(a) Symbiont load in response to assay temperature. Temperature label in grey block indicates the long-term treatment temperature. Lines indicate fitted GAM. (b)

Symbiont load at long-term growth temperature for each long-term treatment. Points indicate means with standard errors.

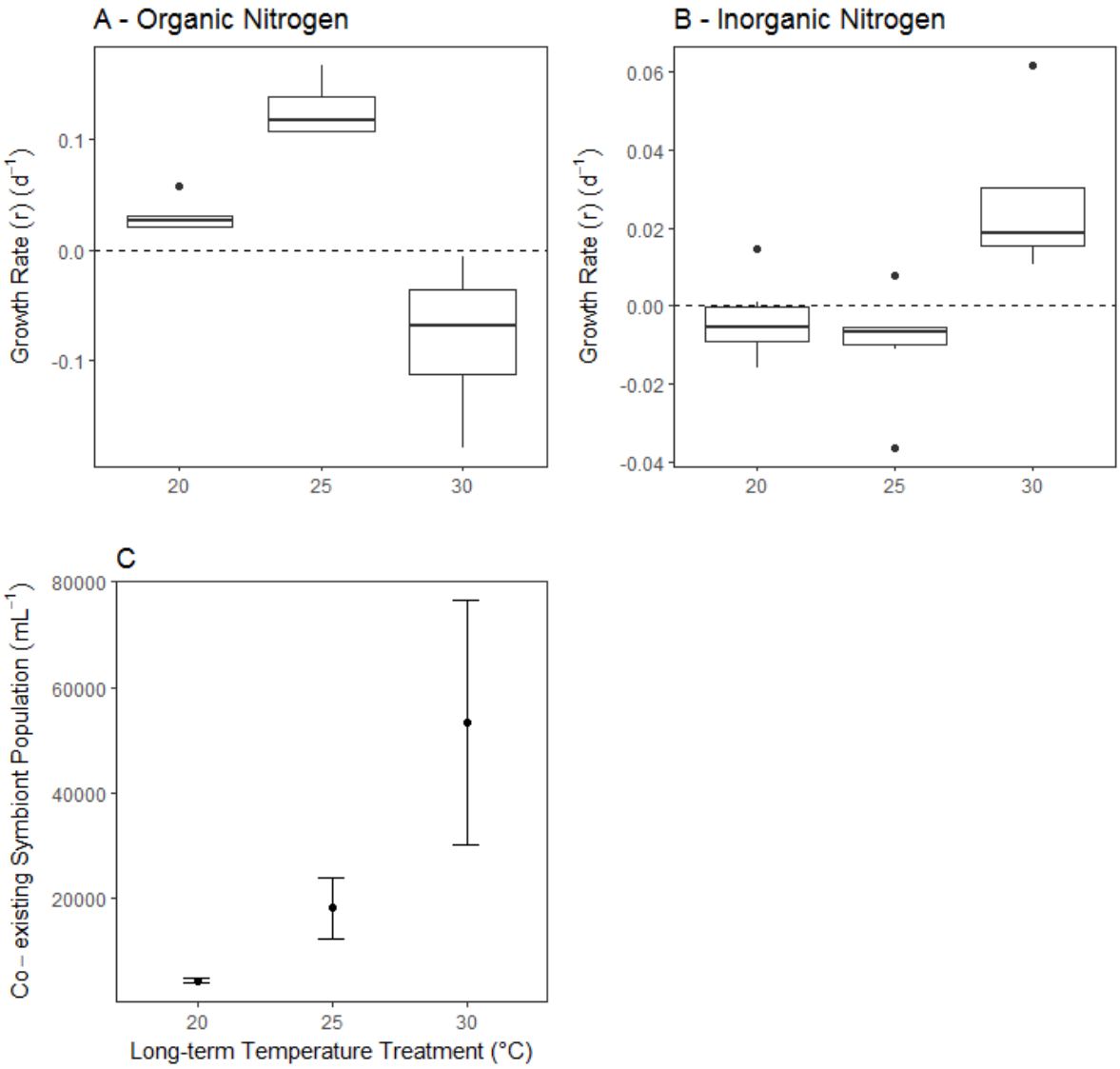


Figure 4: Symbiont nitrogen use and co-existing population size within holobiont cultures

(a) Boxplots (median, first and third quartile and 95% confidence interval of median) for symbionts isolated from each long-term temperature treatment growth rate on organic nitrogen. (b) Boxplots for symbionts isolated from each long-term temperature treatment growth rate on inorganic nitrogen. (c) Symbionts enumerated

living co-existing with holobionts within long-term temperature treatments at the end of the experimental period.

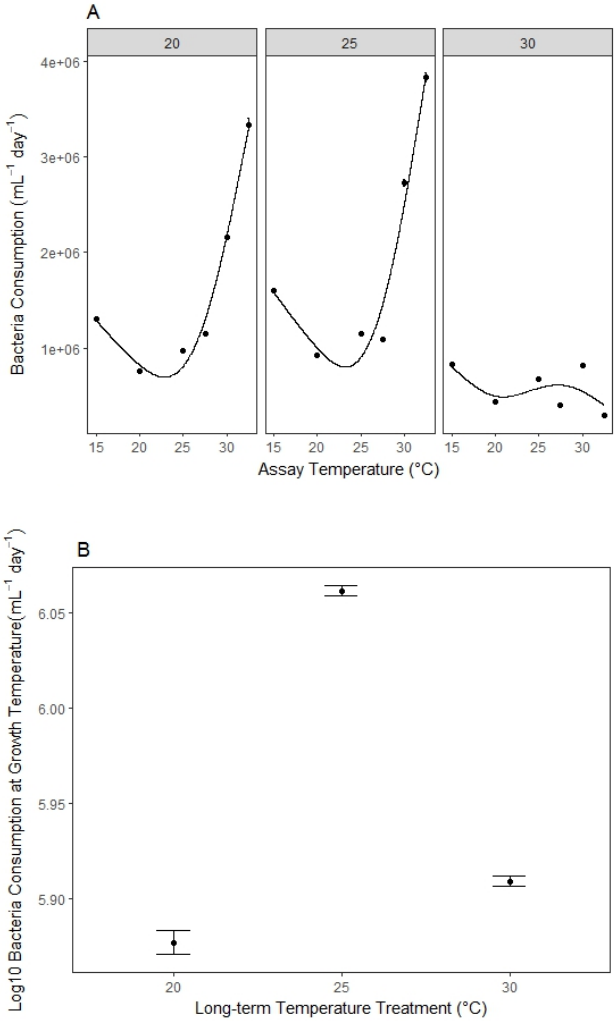


Figure 5: Holobiont bacterivory thermal responses

(a) Bacteria consumption rate per holobiont (mL^{-1} day^{-1} holobiont^{-1}) (bacterivory) in response to assay temperature for each long-term treatment. Temperature label in grey block indicates the long-term treatment temperature. (b) Bacterivory at long-

term growth temperature for each long-term temperature treatment. Log 10 transformation for ease of viewing. Points indicate means with standard errors.

Temperature (°C)	Replicate	Generations Passed	Mean	SE
20	1	60.29705061	55.45614	1.065352
	2	56.52840616		
	3	53.88229007		
	4	54.83438685		
	5	51.79441232		
	6	55.40030004		
25	1	55.23746382	57.65875	0.981175
	2	60.4120758		
	3	58.11190497		
	4	59.28864082		
	5	53.67310481		
	6	59.22931412		
30	1	20.42941407	21.05658	0.215663
	2	20.92039043		
	3	21.58129997		
	4	Extinct before end		
	5	Extinct before end		
	6	21.29523404		

Table 1: Generations passed during experiment (295 days)

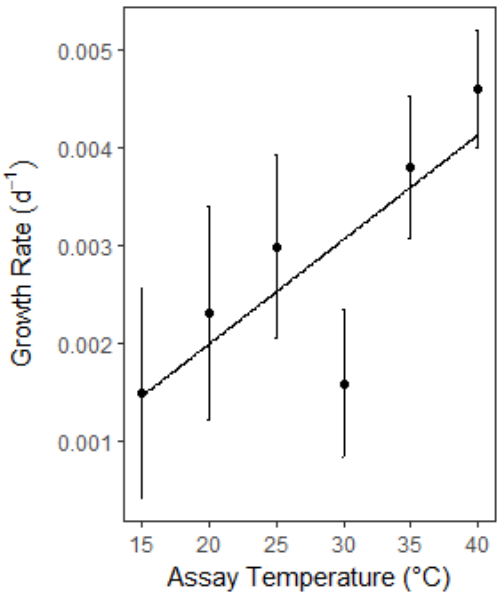


Figure S1: Growth rate of isolated *Serratia marcescens*

Isolated *Serratia marcescens* (provided to holobionts as food source in experiments) growth rate in response to assay temperature. Fitted line shows linear model with assay temperature as main effect. Assay temperature significantly explained growth rate ($F = 6.481$, $df = 1,28$, $p=0.01669$).

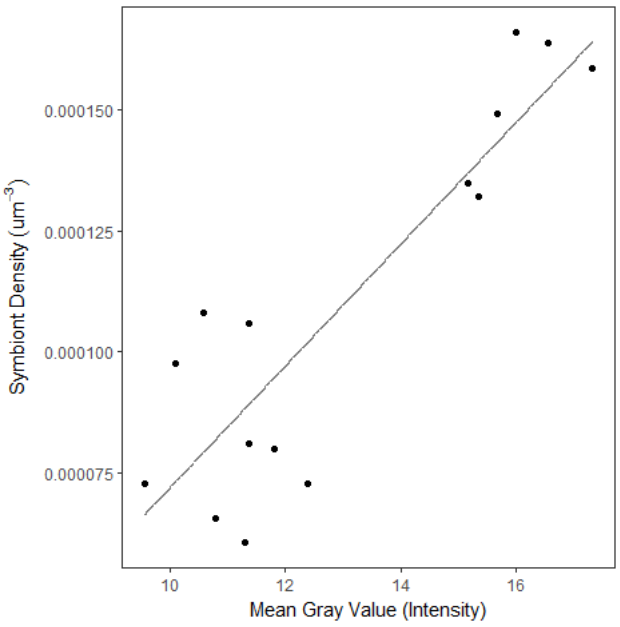


Figure S2: Linear relationship between intensity and symbiont density

Relationship between intensity and symbiont density, used to convert measured intensity values into symbiont density estimates (see Methods) ($F = 45.31$, $df = 1,13$, $p = 1.402e\text{-}^{05}$).

Chapter 5: Discussion

Overview

This thesis presents complimentary research chapters that deal with a number of important research questions relating to the study of the thermal responses of photo-symbiosis, spanning the time scales of seconds (i.e. metabolism) to days (i.e. ecological responses) to months (i.e. evolutionary responses).

First, in chapter 2, we assessed the metabolic responses of *Chlorella spp*. symbionts to temperature, to uncover whether specialisation on host-provided nitrogen ("metabolic integration") impacts their associated thermal responses of key metabolic processes. We tested this idea by examining the impacts of different nitrogen sources on the relative thermal sensitivities of respiration and photosynthesis in symbiotic and free-living *Chlorella spp*. We found that, in free-living *Chlorella* (*Chlorella vulgaris*), nitrogen source influenced metabolic thermal responses and the respiratory cost of growth. Glutamine, in contrast to the other nitrogen sources, resulted in the maintenance of CUE with increases in temperature and results in the lowest respiratory cost of growth. We also found that symbionts were unable to grow on nitrate, but their CUE responses when tested 'in situ' (and grown independently on an amino acid-rich medium) matched those of free-living *Chlorella* on glutamine. Taken together, these observations suggest that the adaptation to symbiosis (via nitrate assimilation degradation and specialisation on amino acids such as glutamine) produces atypical metabolic thermal responses in photo-symbiotic algae.

What does this mean for the response of the holobiont? In chapter 3, we addressed the functional characteristics (symbiont density and host bacterivory) of a tractable photo-symbiosis, the *Paramecium bursaria-Chlorella spp*. association, asking whether the thermal responses of such traits can be simply understood based on those of growth and metabolism alone, or whether 'host responses' – potential mechanisms by which hosts could selectively evict, cull or otherwise regulate their resident symbionts – complicate the predictions based on these fundamental responses. Despite the maintenance of CUE that we observed in symbionts in chapter 2, we found that – when inside the functional holobiont – CUE declines as is predicted in a wide suite of ecological systems with warming (likely as a result of host respiration) (Lopez-Urrutia *et al*. 2006; Anderson-Teixeira *et al*. 2011; Yvon-Durocher *et al*. 2010, 2012). Thus, a number of potential responses to CUE (as discussed in chapter 2) were apparent, and so we asked whether we could understand the functional characteristics (i.e. symbiont load and grazing rate) based on metabolism and growth. We found that both warming and cooling drove increases in bacterivory and reduced symbiont abundances within hosts, suggesting that departures from the thermal growth optimum for symbionts can cause the loss of symbionts and increased heterotrophy in these widespread photo-symbiotic ciliates. Thus, we concluded that the responses of this widespread and ecologically important symbiosis appeared to be explained by the growth response of symbionts.

While insightful, and potentially a general mechanism for symbiosis breakdown (i.e. if general, surpassed thermal tolerance limits of symbionts is likely to lead to

“bleaching” and increased heterotrophy in widespread photo-symbioses), we must readily acknowledge that these responses will almost certainly be influenced by adaptation in the long term (Hoffman & Carla, 2011). What kind of responses could be expected over long-term warming? Does the association possess the capacity for thermal adaptation? What could be the consequences for the wider ecosystem of such adaptation or lack thereof? We went on to address these questions in chapter 4.

There is much debate over whether ecologically-important mutualisms such as photo-symbioses are likely to degrade in response to contemporary climate change (reviewed in Frederickson, 2017 and Kiers et al. 2010); here, degradation refers to a number of potential outcomes: the abandonment of the symbiosis, the reversion to autonomy of one (or both) of the partners, extinction, or progression towards parasitism (e.g. Sachs & Simms, 2006; Kiers et al, 2010). This debate stems largely from the fact that it is inherently difficult to investigate empirically such phenomena, given that such events have often occurred deep in the evolutionary histories of interacting organisms, obscured by the complex evolutionary trajectories they have since followed. Thus, much of our understanding of these potential evolutionary trajectories is derived from theory, modelling, comparative phylogenetics, and extrapolation from short-term ecological responses (see Chapters 1 & 4). While recent reviews of the available evidence have subsequently argued that mutualisms in general are likely to be more robust over evolutionary timescales than once thought (Frederickson, 2017), the field would benefit from direct empirical studies of a range of symbiotic mutualisms. Briefly, this was the rationale for our evolution experiment, again using the tractable photo-symbiosis, *P. bursaria-Chlorella spp*.

Following ~10.5 months (and approximately ~21 generations) of experimental warming, we observed apparently conflicting results: symbionts appeared to gain the ability to utilise nitrate in the growth medium, 'switching' from host-provided nitrogen sources normally provided in photo-symbiosis and arguably becoming autonomous. Simultaneously, we observed the thermal adaptation of growth in warm-treated holobionts; these hosts continued to associate with symbionts, evidencing the changed thermal niche of the photo-symbiosis in response to long-term warming. This is suggestive of two disparate evolutionary trajectories, likely following during the course of the experiment: that which drives autonomy and which drives the maintenance of symbiosis. We suggest ways that this outcome could 'collapse' when within the context of real-world ecosystems; here, the capacity for symbiont autonomy is likely to be harshly selected against, owing to the presence of predators, competitors, and specialist viruses. For example, a key benefit of photo-symbiosis for symbionts in natural environments is protection from viral threats (Kodama & Fujishima, 2009 and references therein); in native freshwater, the titre of symbiont-specific viruses can reach strikingly-high levels and viruses are thus likely to be a significant threat for autonomous symbionts (Grimsley et al. 2012).

Taken together, the research in this thesis suggests that symbiont physiology directly underpins the responses of photo-symbiosis to warming in the short term, in answer to our first research question. Second, we present evidence for divergent trajectories followed by photo-symbioses with long-term warming; thus, further work that investigates whether these outcomes would be possible in nature are now of paramount importance and will help predict the likely fate of photo-symbiosis in a warmer world.

Summary of Key findings

Chapter 2: The metabolic thermal responses of Chlorella spp. symbionts

- In free-living *Chlorella* (*C. vulgaris*), nitrogen source influenced metabolic thermal responses and the respiratory cost of growth:

    - The nitrogen source significantly affected the thermal response of GPP but not R.

    - Glutamine, in contrast to the other nitrogen sources, resulted in the maintenance of CUE with increases in temperature and the lowest respiratory cost of growth across all temperatures.

- Compared to published values in the literature, the symbiont displayed increased temperature sensitivity of GPP and reduced sensitivity of R.

- We also found that symbionts were unable to grow on nitrate, but their CUE responses when tested ‘in situ’ (or grown independently on an amino acid-rich medium) matched those of free-living *Chlorella* on glutamine.

- Taken together, these observations suggest that the adaptation to symbiosis (via nitrate assimilation degradation and specialisation on amino acids such as glutamine) produces atypical metabolic thermal responses in photo-symbiotic algae.

Chapter 3: Short-term responses to warming

- In holobionts, CUE declined with warming, since R was more sensitive to temperature than GPP. This suggests that, despite the observed lack of CUE sensitivity in symbionts in Chapter 2, when within the context of the functional holobiont, the outcome of both symbiont and host metabolism combined is that CUE declines. Thus, temperature reduces the theoretical maximum fraction of carbon available for translocation from symbionts after accounting for the respiration of the host and its symbionts. This thermal constraint on CUE is likely to disrupt the metabolite trade at the heart of the photo-symbiosis, potentially reducing the 'value' of symbionts to hosts.

- Does this CUE response invoke host regulation mechanisms? Such reduction in the fraction of symbiont photosynthate offered via translocation might be expected to incur host regulatory mechanisms that are thought to regulate symbiont abundances and selectively remove inefficient, 'costly' symbionts. However, we found that symbiont abundance within cells broadly matched the growth response of independently grown symbionts; thus, there was no clear evidence for host control. This finding (or lack thereof) is in agreement with recent work suggesting that growth dynamics are important determinants of symbiont abundance within hosts (i.e. the thermal response of symbiont growth explains the observed symbiont abundance within host response).

- Do hosts up-regulate bacterivory in response to warming, as theory – backed up with experimental and observational evidence – would predict? Bacterivory indeed varied across the temperature gradient, and increased at the highest temperatures, but this response appeared to largely be the inverse of the symbiont abundance and symbiont growth responses. Specifically, bacterivory rate remained low at intermediate temperatures (where symbiont abundance and growth was high) while rapidly increasing with warming and cooling relative to this intermediate range (where symbiont abundance and growth declined). The simplest explanation for this pattern is that bacterivory was simply constrained to increase where symbiont abundance was low as a result of low symbiont growth rate (i.e. hosts are 'secondarily' heterotrophic). As we discuss in the chapter, this could be explained by a physical volume trade-off within the host cell; volume occupied by symbionts potentially leaves less space for the processing and transport of food vacuoles. This is perhaps particularly likely, given that symbionts are also encapsulated in individual membranes (sharing the same system that prey items are engulfed by) – a host activity that could directly reduce the available membrane constituents to phagotrophy.

Chapter 4: Long-term responses to warming

- Holobionts produced an adaptive growth response in the long-term warming (~10.5 months; ~21 generations) treatment: the optimum temperature for

growth (T_{opt}) shifted towards the high temperature and was significantly higher than the T_{opt} measured for the long-term ambient (control) treatment.

- Strikingly, symbionts, mechanically extracted from holobionts such that we could run tests, appeared to 'switch' their nitrogen source capability. Whereas symbionts from the ambient and the cooled treatments were able to grow on organic nitrogen (i.e. in a medium containing bacto-peptone; a common growth medium component that contains mainly oligopeptides and free amino acids) but not inorganic nitrogen (i.e. in a medium containing nitrate as the sole nitrogen source), symbionts from warm-adapted holobionts (i.e. from the long-term warming treatment) showed the opposite capability. These symbionts could grow only on inorganic, and not organic, nitrogen.

- CUE was significantly lower across a thermal gradient in warm-adapted holobionts compared to the ambient and cooled treatments. In fact, CUE was ~0 at the long-term growth temperature, suggesting that there is likely to be an extremely strict supply of photosynthates available for translocation to hosts during metabolite trade under these conditions. This raises the question, why did hosts continue to harbour symbionts at this temperature? One intriguing possibility is that hosts were unable to exert control over their symbiont populations, since one regulation mechanism is organic nitrogen restriction. We showed that symbionts appeared to evolve to utilise inorganic nitrogen (and they were unable to grow on the organic nitrogen source). This suggests that the host control mechanism became incompatible with symbiont physiology.

- NPP was significantly reduced in the warm-adapted holobionts across the temperature gradient and also approached ~0 at long-term growth temperature.

- Similarly, bacterivory was also significantly reduced in the warm-adapted holobionts. Here, bacterivory displayed a strikingly different thermal response across the temperature gradient compared to the ambient and cooled treatments: it remained relatively low, and invariant, with temperature. This contrasts with the thermal response for the other two treatments, where bacterivory sharply increased with a high degree of warming.

- Taken together, the reduction in NPP and bacterivory suggest that the ecological function of the holobiont in the context of wider ecosystems could change in response to long-term warming. Currently, photo-symbiosis is responsible for approximately half of all marine photosynthesis (Baldauf, 2008), likely represents (together with other photo-mixotrophs; i.e. organisms that combine phototrophy with heterotrophy) an important source of grazing in aquatic ecosystems (Berninger et al. 1992; Unrein et al. 2007; Zubkov & Tarran, 2008; Hartmann et al. 2012) and provides important ecosystem services in both marine and freshwater ecosystems (reviewed in Not et al. 2006). Thus, these findings have clear relevance for these ecosystems, where photo-symbioses that no longer perform their important ecological ‘functions’ could have detrimental impacts on biodiversity and influence whether such ecosystems behave as net carbon sources or sinks.

The host is in control: Or is it?

There is a developing view that the *P. bursaria-Chlorella spp*. association and other photo-symbioses could be considered exploitative, whereby the host retains 'control' over, and perhaps even parasitizes, its symbionts; this view clearly differs from the widely-held belief that these symbioses are mutualistic (Lowe et al. 2016; Sørensen et al. 2019; Minter et al. 2018; Dean et al. 2016). However, a number of findings in this thesis, regarding how the system responds to warming, challenge this view. For example, despite the suite of regulatory mechanisms thought to be employed by *P. bursaria* in order to control and even 'exploit' their symbionts (e.g. Kodama & Fujishima, 2008; Kadono et al. 2004; Lowe et al. 2016; He et al. 2019; see Introduction; Box 1), we found that symbiont growth appeared to drive the ecological responses of the *P. bursaria-Chlorella spp.* symbiosis, and there was no clear evidence for any such host-derived regulation. For example, in previous work on light intensity, Lowe et al. 2016 found that the host likely restricts symbiont load at high light irradiances (where each symbiont is more 'valuable' as a result of increased productivity, and the host therefore requires fewer to meet the same carbon demands) and might also restrict symbiont load in the dark, where symbionts no longer provide a photosynthetic benefit. However, note that the autonomous growth rate of symbionts increased monotonically with light irradiance in this past work (Lowe et al. 2016); thus, an alternative way of interpreting these data would be to posit that symbiont growth potential, rather than host control, restricts symbiont abundance in the dark. Thus, while the host interests and effects of symbiont growth are probably aligned in this scenario (i.e. symbiont growth rate is low where hosts require few/no symbionts), this work is in broad agreement with the research in this

thesis on short-term impacts of a different abiotic factor (i.e. temperature). Here, symbiont load appeared to be restricted directly by symbiont growth potential; note that this occurred both where the host's interests are theoretically aligned (i.e. each symbiont is theoretically more 'valuable' as a result of high CUE at cooler temperatures, and the host therefore probably requires fewer to meet the same carbon demands) and where they may not be (i.e. at higher temperatures, more symbionts could theoretically be required to meet the same carbon demands as a result of low CUE; this would presumably reach some critical threshold whereby further warming renders symbionts costly and thus this requirement would collapse).

A recent study that interrogates the actual cost/benefit balance associated with this photo-symbiosis, by comparing the fitness of hosts with and without symbionts in response to temperature in the short term, has been conducted (Salsbery and DeLong, 2018). Intriguingly, this work shows that symbiosis is beneficial for *P. bursaria* at cooler temperatures and becomes costly at higher temperatures. The authors suggested that this is because alga-free *P. bursaria* can make effective use of heterotrophy (i.e. bacterivory) at higher temperatures while symbiotic *P. bursaria* benefit more from algae at cooler temperatures. This thesis potentially provides support for both of these suggestions. Firstly, our experiments revealed that bacterivory followed the inverse of the intracellular symbiont abundance thermal response, and we speculated that a simple explanation could be that there is a phagotrophy:symbiont encapsulation trade-off (imposed by host volume and membrane constituent supply; see Chapter 3). Thus, bacterivory could be limited in symbiotic hosts (relative to autonomous hosts) due to this simple constraint. Second, we show that holobiont CUE declines with warming, providing a simple reason for

the transitioning of the symbiosis from being beneficial to costly for hosts with temperature; low CUE is likely to constrain the relative balance of photosynthates that the algae are able to provide their hosts and thus warming could erode their photosynthetic benefit (see Chapter 3). A short-term study examining the cost/benefit balance associated with a coral-zooxanthellae photo-symbiosis has also been conducted (Baker et al. 2018). In this work, the authors show that warming drives the association to become costly for hosts as a result of metabolic responses (declining holobiont NPP and increasing host R), in conceptual agreement with our results, which shows that holobiont CUE declines across a broad temperature gradient. This coral research also shows that the host likely pays the respiratory costs of warming, and not symbionts (Baker et al. 2018). Intriguingly, this finding also links with this thesis. Comparing holobiont and 'in situ' symbiont metabolism (measured in Chapters 3 and 2, respectively), it is likely the hosts that pay the respiratory costs of warming; autonomous symbionts had similar thermal sensitivities of GPP and R while R is more thermally sensitive in the holobiont. Furthermore, glutamine (which is the nitrogen source provided by hosts to symbionts) resulted in lower maintenance R costs and relatively higher GPP compared to R sensitivity in the alga *C. vulgaris*, suggesting that the metabolic costs of 'nitrogen handling' could also represent an example of an asymmetric metabolic cost balance in the photo-symbiosis (see Chapter 2). Interestingly, these metabolic findings again challenge the view that the host exploits the algae in photo-symbiosis.

Why was the symbiont load thermal response apparently different for the long-term ambient population in the evolution experiment detailed in Chapter 4, compared to that of holobionts at the same temperature in Chapter 3? A key reason is likely to be

light intensity, which differed between these separate experiments. Although we did not measure autonomous symbiont growth across a temperature gradient for each temperature treatment in the evolution experiment, note that the idea that symbiont growth underpins symbiont load appears to hold: symbionts grew best at 25°C, worse at 20°C, and worst at 30°C (long-term temperatures), mirroring the apparent differences between the intercept of the symbiont load responses at each (long-term) temperature (see Chapter 4), suggesting that symbiont growth constrains symbiont load. The view that thermal impacts on symbiont growth underpins symbiont load in this thesis carries an obvious link to the bleaching response of corals, where it is largely thought to be pathological impact of warming that drives the loss of symbionts from hosts (Lesser, 2011; although see work on the intensely debated 'adaptive bleaching' hypothesis for an alternative view, which posits that the bleaching response is ultimately an adaptive one and not a pathological one; Buddemeier & Fautin, 1993) and the loss or switching of symbionts in lichens (Rolshausen et al. 2018). Clearer still is the link to work in other symbioses which also shows that temperature can directly influence symbiont abundance within hosts, for example in stink bugs (Kashkouli, Fathipour & Mehrabadi, 2019; Kikuchi et al 2016; Prado et al. 2010) and beetles (Six & Bentz, 2007). Indeed, the sensitivity of symbionts to environmental perturbation has been referred to as the "Achilles heel" of insect-symbiont mutualisms (Wernegreen, 2012). More broadly, this suggests that – at least in the short term – asymmetric responses of hosts and symbionts are likely to be a major driver of symbiosis breakdown, in agreement with a suite of recent studies on environmental stressors and symbiosis (e.g. Harrison, 2000; Jevanandam, Goh & Corlett, 2013; Wernegreen, 2012; Kashkouli, Fathipour & Mehrabadi, 2019; Kikuchi et al 2016; Prado et al. 2010; Six & Bentz, 2007; Dysthe,

Bracewell & Six, 2015; Robbirt et al. 2014; Warren & Bradford, 2014; Warren, Bahn & Bradford, 2011; Sutton et al. 2018). Are algae the “Achilles heel” of photo-symbioses?

Other findings that could usurp hosts as the ‘controllers’ in the *P. bursaria-Chlorella spp*. association came from our evolution experiment (Chapter 4). We found that warm-adapted holobionts were strikingly less productive and had a CUE and NPP of ~0. Although other benefits of symbiosis for hosts that we did not study could be at play (e.g. photo-protection; Summerer et al. 2009), it is unclear why hosts should harbour symbionts when their photosynthetic benefit is likely to be very low at best. We suggest that one reason for this might be that symbionts evolved to ‘escape’ one method by which hosts have been previously shown to regulate symbiont abundance: nitrogen provisioning (Lowe et al. 2016; He et al. 2019). This could be interpreted as symbionts evolving to evade host control measures and become more autonomous (and potentially become commensal or even parasitic when inside the host as a result of their CUE response). Intriguingly, this suggests that the symbiont dependency on hosts that has probably evolved as a result of specialisation on glutamine (that we explored in Chapter 2) is apparently reversible, in contrast with the idea that adaptation to symbiosis may commonly restrict the breakdown of symbiosis (Werner et al. 2018). A high degree of symbiont dependency (and thus strict vertical transmission of symbionts) should align symbiont:host interests and function to prevent symbiont ‘cheaters’ evolving – symbionts that do not adhere to a mutualistic exchange of services – in symbiosis (see Foster et al. 2017; Sachs et al. 2004; Edwards, 2009), yet if this dependency is flexible, symbiont cheating could presumably more readily emerge. This brings into question the stability of the

association over evolutionary timescales in the face of climate change. In other words, if symbionts are able to circumvent host sanctions and 'undo' adaptations that drive dependency on hosts in response to warming, then photo-symbiotic hosts may be less able to restrict the emergence of symbiont 'cheat' genotypes in a warmer world.

Going green: Can warming make the photo-symbiosis 'more autotrophic'?

Although we did not actually measure carbon uptake via autotrophic (i.e. GPP) and heterotrophic (i.e. bacterivory) processes and thus the following is speculative, the increase in symbiont density and the decrease in bacterivory that we observed in the short-term experiment (see Chapter 3) could suggest that the *P. bursaria-Chlorella spp*. association became increasingly relatively autotrophic with sub-pathological warming (i.e. between 15°C and ~30°C). This observation contrasts with previous work on a different form of mixotroph (i.e. chloroplast-bearing protists capable of heterotrophic grazing), in which warming has been shown to promote heterotrophy (Wilken et al. 2013), and with general predictions/observations that the rates of heterotrophic processes increase more rapidly with temperature than photosynthetic autotrophy (e.g. Wilken et al. 2014; Lopez-Urrutia et al. 2006; Rose & Caron, 2007; Allen et al. 2005). This apparent contrast between photo-symbioses and mixotrophy in free-living protists (Wilken et al. 2013) seems most likely a function of host-symbiont ecological dynamics, which may allow for a different degree of plasticity; indeed, flexibility in the relative abundance of photosynthetic and heterotrophic machinery may subvert the general prediction that warming should promote a

heterotrophic lifestyle, even in single organisms (Wilken et al. 2013). In the context of photo-symbiosis, the relative abundance of autotrophs (i.e. symbionts) and heterotrophs (i.e. hosts) represents a simple ecological adjustment that can counteract the thermodynamic tendency for warming to promote heterotrophy. This process, observed in photo-symbiosis in the current study, parallels a recent study showing that changes in autotrophic biomass resulted in maintenance of GPP despite temperature-induced changes in free-living phytoplankton communities (Padfield et al. 2017). In a wider context, a broad range of other studies have shown that the impacts of metabolic thermal constraints may be modified by a number of short-term ecological adjustments, such as species turnover (Karhu et al. 2014), phenotypic plasticity (Luo et al. 2007) and resource depletion (Melillo et al. 2002).

The bacterivory and symbiont load responses appeared to be similar in the ambient holobiont (i.e. at the same temperature) in the evolution experiment (detailed in Chapter 4): symbiont load peaked and fell while bacterivory troughed and increased with warming. However, the optima/minima of the responses appeared to be different (e.g. the thermal optimum for symbiont load appeared to peak earlier in the evolution experiment): this is likely to be a result of the different light intensities across these two separate experiments, as discussed previously. This is important, since the locations of the peaks of the thermal response curves define whether the same degree of warming might favour autotrophy or heterotrophy, if we are to build upon the ideas presented above. This emphasises the importance of a multifactorial approach in understanding the real-world impacts of warming on the photo-symbiosis for its wider ecosystems. Regardless of the short term response, we found that adaptation to warming appeared to strongly down-regulate the bacterivory

thermal response; warm-adapted holobionts displayed a relatively low and stable bacterivory rate across all tested temperatures. Furthermore, warm-adapted holobionts displayed a strikingly low NPP. What impacts could such changing bacterivory and productivity have on natural ecosystems in a warmer world? They could be severe: microbial photo-symbioses are key sources of production, bacterivory and species abundance (Berninger et al. 1992; Unrein et al. 2007; Zubkov & Tarran, 2008; Hartmann et al. 2012; Sonntag et al. 2011; Summerer et al. 2008; Sanders, 1991, 2011; Decelle, Colin & Foster, 2015; Baldauf, 2008). Thus, the relative balance between autotrophy and heterotrophy in such associations – and the degree of warming that can cause shifts in this balance – is likely to be important in assessing the ecological consequences of climate change.

Evolutionary trajectories of photo-symbioses: Autonomy and maintenance?

Predictions about the future state of photo-symbioses and important ecosystem services that rely upon them such as coral reefs are highly dependent on the capacity of photo-symbioses to adapt in the face of warming. However, many ecologically-important symbioses including the coral-symbiont association feature hosts which are slow growing and difficult to cultivate (Chakravarti & van Oppen, 2018), and as such evolutionary responses have been difficult to study directly, despite evidence for local adaptation that has occurred over unknown timescales in nature (e.g. Weis, 2010). Notably, while the thermal responses of autonomous symbionts of corals have been cultivated and studied over multiple generations – research which has revealed that autonomous symbionts of corals can rapidly adapt

to warming (Chakravarti & van Oppen, 2018), paralleling the conclusions of other studies on free-living phytoplankton (Padfield et al. 2016; Schaum et al. 2017) – no studies involving experimental evolution have investigated the responses of any photo-holobiont to warming (as far as we are aware). This is despite the fact that such studies examining thermal adaptation under monitored conditions have been conducted in other symbioses such as squid-bacteria associations (e.g. Cohen et al. 2019). As a result, how photo-symbioses could respond to global warming is uncertain, especially regarding the timescales under which adaptation could occur (e.g. Baker et al. 2008); yet this issue remains at the forefront of environmental concerns (IPCC, 2018; 2019). In our evolution experiment, we yield possible responses of photo-symbioses in a warmer world by using our tractable, microbial association.

Intriguingly, we found that warming produced an adaptive growth response in holobionts but also appeared to drive the evolution of increased autonomy in symbionts, suggesting that two disparate evolutionary trajectories were followed. According to phylogenetic evidence, reversion to autonomy appears to be the most likely source of breakdown in symbiotic mutualisms (Sachs and Simms, 2006). This might be particularly common in nutritional mutualisms when one partner can acquire the same benefit from the environment (Werner et al. 2018), which carries a clear link to our work: the symbionts appeared to turn to inorganic nitrogen contained in the growth medium, as opposed to that which is provided in symbiosis (i.e. glutamine; see Chapter 4). Furthermore, the simultaneous maintenance of symbiosis and reversion to autonomy of one of the partners appears to be a potential outcome for photo-symbioses over evolutionary timescales. We argued that whether such an

outcome could occur in nature will likely depend on a multitude of other factors, such as the presence of viruses like PBCV-1 that cause mortality to free-living *Chlorella* and can reach high titres in native freshwater (Kodama & Fujishima, 2009 and references therein; Grimsley et al. 2012; see Chapter 4).

Loss of holobiont productivity with long-term warming: A cause for concern?

Photo-symbiotic mutualisms – including the numerous examples in the planktonic realm – are responsible for ~50% of all marine photosynthesis (and thus a significant portion of the total global carbon budget; Baldauf, 2008), and much of both freshwater and marine biodiversity (Bailly et al, 2014; Davy et al, 2012; Weis, 2010; Sonntag et al. 2011; Summerer et al. 2008; Sanders, 1991, 2011; Decelle, Colin & Foster, 2015). Accordingly, understanding the likely future impacts of temperature on photo-symbiotic mutualisms and their wider ecosystems represents an ongoing major challenge for ecologists. Strikingly, per capita NPP was close to zero at growth temperature under the long-term warming condition in our evolution experiment. This could indicate that rates of global photosynthesis are at risk as a result of the impacts of global warming on photo-symbioses. Thus, while the holobiont can seemingly rapidly adapt to warming (evidenced by maximum growth rate and thermal optimum for growth), with potentially encouraging implications for the persistence of the symbiosis in a warmer world (although the symbionts also appear to re-gain independence; see above), our data also show that the ecological function of the photo-symbiosis is likely to be strikingly different in a warmer world. These findings raise concerns about what the consequences of thermal adaptation in

photo-symbioses could be on the ecosystem services provided (see Baker et al. 2008).

Organic nitrogen and carbon-use efficiency: A potential spin-off?

It has long been known that photorespiration limits the efficiency of photosynthesis, and it is thus unsurprising that a large research effort has been made to eliminate or down-regulate this process in crops, for example via genetic engineering (reviewed in Betti et al. 2016). However, it is now known that photorespiration also plays an important role in plant metabolism; one key recent discovery is that it appears to be linked to the assimilation of nitrates in the nitrogen assimilation pathway (Rachmilevitch et al. 2004; Bloom, 2014). Thus, the nitrogen source is likely to be important in determining photorespiration and thus photosynthetic rates in agriculture. Currently, nitrates are normally the most abundant form of nitrogen provided to crops, yet there is increasing interest in understanding the importance of organic nitrogen in agricultural systems (Dion et al. 2018; Reganold and Wachter, 2016). As knowledge about (and potentially greater incorporation of) organic nitrogen increases, a key question is therefore, could organic nitrogen have impacts on photosynthetic efficiency in crops? Critically, this question should be addressed in the context of global warming, since it will be increasingly important to understand how changing practices in agriculture could influence the responses of crops to warming (e.g Mendelsohn et al. 1994). Thus, agricultural science could benefit from an understanding of the influence of nitrogen source on primary producer metabolism across a temperature gradient in a wide suite of organisms.

In our tests on *C. vulgaris*, conducted as part of our research detailed in Chapter 2, nitrogen source significantly affected the temperature response of GPP but not of R. CUE declined rapidly with temperature on nitrate and ammonium, but not on glutamine (where it was in fact subject to a shallow increase). This suggests that GPP thermal sensitivity is increased on glutamine compared to nitrates and ammonium, providing a temperature-dependent benefit to CUE. Intriguingly, since photorespiration is thought to limit the thermal sensitivity of GPP (Barton et al. 2018), it appears that simply providing glutamine could decrease photorespiration rate in *C. vulgaris.* This is harmonious with a strategy whereby photorespiration – which has been shown to be important in the assimilation of nitrates – is down-regulated when it is not necessary (i.e. when organic nitrogen is provided instead of nitrates, and nitrate assimilation is thus not required; see Rachmilevitch et al. 2004; Bloom, 2014). If general, this metabolic response suggests that the thermal sensitivity of GPP in crops could be increased simply by providing organic nitrogen; this means that the resultant GPP increase would be greater at higher temperatures – this is of clear relevance for addressing productivity concerns in a warmer world (e.g 2018; Mendelsohn et al. 1994). In summary, this work suggests that an increased use of organic nitrogen could theoretically improve photosynthetic efficiency, especially at warmer temperatures. While the *Chlorella* genus is relevant to agriculture and industry per se (e.g. Belasco, 1997), it is clear that future work could investigate this potential in other organisms such as crop plants and our work could be interpreted as a simple first step towards understanding how organic nitrogen might impact agriculture.

Closing remarks

In this thesis, we aimed to address the specific research questions: Firstly, can we understand the responses of a microbial photo-symbiosis based on metabolism and growth dynamics? Second, how will microbial photo-symbioses respond to warming, and what could the consequences for the wider ecosystems be – over long (evolutionary) time scales? It is my hope that we have succeeded in addressing these questions. Taken together, the research in this thesis suggests that symbiont physiology simply and directly underpins the responses of photo-symbiosis to warming in the short term, in answer to our first research question. Second, we present evidence for divergent trajectories followed by photo-symbioses with long-term warming; thus, further work that investigates whether these outcomes would be possible in nature are now of paramount importance and will help predict the likely fate of photo-symbiosis in a warmer world.

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Appendix: Statistical Outputs & Model Selection Tables

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
1	8	-19.83961	-7.17146	17.91980			
2	6	-15.57282	-6.07170	13.78641	1 vs 2	8.26679	0.0160
3	4	-12.06833	-5.73425	10.03416	2 vs 3	7.50449	0.0235
4	3	63.51883	68.26938	-28.75941	3 vs 4	77.58715	<.0001

(A) Selection table for *C. vulgaris* thermal photosynthesis response explained by nitrogen source. **1=global**, 2=no interaction effect, 3=no main effect, 4=null.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
1	8	65.34091	78.00906	-24.67045			
2	6	64.11379	73.61490	-26.05689	1 vs 2	2.77288	0.2500
3	4	62.73292	69.06699	-27.36646	2 vs 3	2.61913	0.2699
4	3	98.90813	103.65869	-46.45406	3 vs 4	38.17521	<.0001

(B) Selection table for *C. vulgaris* thermal respiration response explained by nitrogen source. 1=global, 2=no interaction effect, **3=no main effect**, 4=null.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
1	8	-143.1306	-130.4625	79.56531			
2	6	-135.0180	-125.5169	73.50900	1 vs 2	12.112627	0.0023
3	4	-133.9665	-127.6324	70.98324	2 vs 3	5.051505	0.0800
4	3	-130.7261	-125.9756	68.36306	3 vs 4	5.240375	0.0221

(C) Selection table for *C. vulgaris* thermal carbon-use efficiency response explained by nitrogen source. **1=global**, 2=no interaction effect, 3=no main effect, 4=null.

Chapter 2: Table S1: Linear mixed-effect model selection tables

Selection tables for linear mixed-effect models for a) *C. vulgaris* thermal photosynthesis response b) thermal respiration response and c) carbon-use

efficiency thermal response. In all cases, we set up the models to examine the impact of nitrogen source on the shape of the thermal response (i.e. the nitrogen source: assay temperature interaction) and on the intercept of the thermal response curve (i.e. the nitrogen source main effect). Models ranked by AIC. All models include a random effect of replicate nested within nitrogen source.

```
formula = CUE ~ 1 + assayT * medium

Residuals:
      Min        1Q    Median        3Q       Max
-0.052648 -0.016354 -0.000358  0.014209  0.080465

Coefficients:
```

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	0.974399	0.029056	33.535	<2e-16	***
assayT	-0.002370	0.001263	-1.876	0.0704	.
mediumglutamine	-0.102557	0.041091	-2.496	0.0183	*
mediumnitrate	0.016069	0.041091	0.391	0.6985	
assayT:mediumglutamine	0.003696	0.001787	2.069	0.0473	*
assayT:mediumnitrate	-0.002220	0.001787	-1.243	0.2235	

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.02936 on 30 degrees of freedom
Multiple R-squared:  0.4597,   Adjusted R-squared:  0.3697
F-statistic: 5.106 on 5 and 30 DF, p-value: 0.001668
```

Chapter 2: Table S2: *Chlorella vulgaris* CUE linear model coefficients

Linear model coefficients table for *C. vulgaris* CUE explained by assay temperature and medium linear model, which included the interaction effect between these two explanatory variables.

(A) Respiratory cost of growth

```
Model 1: (resp_per_unit_growth) ~ 1 + assayT * medium
Model 2: (resp_per_unit_growth) ~ 1 + assayT + medium
Model 3: (resp_per_unit_growth) ~ 1 + assayT
```

	Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)	
1	21	6.4619e-07					
2	23	7.9401e-07	-2	-1.4782e-07	2.402	0.115	
3	25	3.0341e-06	-2	-2.2401e-06	36.400	1.497e-07	***

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

(B) Symbiont growth rate on different nitrogen sources

Model 1: Growth_Rate_Estimate ~ medium
Model 2: Growth_Rate_Estimate ~ 1

	Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	6	0.001829				
2	8	0.312289	-2	-0.31046	509.31	2.008e-07 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(C) Freshly-liberated/‘in situ’ symbiont CUE

Model 1: CUE ~ assayT
Model 2: CUE ~ 1

	Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	31	0.33948				
2	32	0.34487	-1	-0.0053974	0.4929	0.4879

Chapter 2: Table S3: Linear model selection tables

Linear model selection tables for a) respiratory cost of growth b) symbiont growth rate on different nitrogen sources and c) freshly-liberated/’in situ’ symbiotic *Chlorella* CUE. Global model in a) is constructed as the dependent variable explained by assay temperature and medium, including the interaction effect between these two explanatory variables. Reduced models were then constructed and were compared to the global model via ANOVA tests. Global model in b) is constructed as the dependent variable explained by medium and is compared to null model via ANOVA testing. Global model in c) is constructed as the dependent variable explained by assay temperature and is compared to null model via ANOVA testing.

(A) Respiratory cost of *C. vulgaris* growth on different nitrogen sources

Medium

	diff	lwr	upr	p adj
glutamine-ammonium	-0.0005517218	-0.0007710707	-0.0003323728	0.0000058
nitrate-ammonium	0.0001050021	-0.0001143468	0.0003243510	0.4657959
nitrate-glutamine	0.0006567239	0.0004373750	0.0008760728	0.0000004

(B) Symbiont growth rate on different nitrogen sources

Medium

	diff	lwr	upr	p adj
bactopeptone-nitrate	0.4503485	0.4066118	0.4940851	3.00e-07
host extract-nitrate	0.2810296	0.2372930	0.3247662	2.50e-06
host extract-bactopeptone	-0.1693189	-0.2130555	-0.1255822	5.32e-05

Chapter 2: Table S4: Tukey's multiple comparisons of means tables

Tukey's multiple comparisons of means tables displaying significant difference tests between a) *C. vulgaris* respiratory cost of growth on different nitrogen sources and b) symbiont growth rate on different nitrogen sources (see respective sections in Results and Methods; Chapter 2).

Global model call: gam(formula = CUE ~ s(assayT, k = 6), data = working_df
2, method = "REML")

Model selection table

	(Int)	s(asT,6)	df	logLik	AICc	delta	weight
2	**0.5106**	**+**	**6**	**57.112**	**-96.3**	**0.00**	**1**
1	0.5106		2	23.508	-42.6	53.66	0

Chapter 2: Table S5: Model selection for generalised additive model fitting independently-grown symbiont carbon-use efficiency to temperature

GAMs constructed to analyse the main effect of assay temperature on CUE, allowing for a non-linear response. Candidate models ranked by AICc.

(A) Carbon-use efficiency

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
Global	1	11 -36.61210	-16.02889	29.30605			
No interaction effect	2	7 -41.44625	-28.34784	27.72312	1 vs 2	3.165852	0.5305
Assay temperature only	3	5 -34.85692	-25.50091	22.42846	2 vs 3	10.589330	0.0050

(B) Net primary productivity

	Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
Global	1	11	-213.8829	-193.2997	117.94147			
No interaction effect	2	7	-197.7115	-184.6130	105.85573	1 vs 2	24.17149	1e-04
Assay temperature only	3	5	-186.2379	-176.8818	98.11893	2 vs 3	15.47359	4e-04

Chapter 4: Table S1: Model selection for linear mixed effects models

Selection tables for linear mixed-effect models for a) carbon-use efficiency and b) net primary productivity. In all cases, we set up the models to examine the impact of long-term temperature treatment on the shape of the thermal response (i.e. the long-term temperature: assay temperature interaction) and the intercept of the thermal response curve (i.e. the long-term temperature main effect). Models ranked by AIC. Random effects were determined at the level of replicates nested within long-term treatment.

Symbiont growth on organic nitrogen

Analysis of Deviance Table

Model 1: Growth_Rate_Estimate ~ Evolved_Temp
Model 2: Growth_Rate_Estimate ~ 1

	Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	13	0.020397				
2	15	0.122778	-2	-0.10238	32.626	8.569e-06 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Symbiont growth on inorganic nitrogen

Analysis of Deviance Table

Model 1: Growth_Rate_Estimate ~ Evolved_Temp
Model 2: Growth_Rate_Estimate ~ 1

	Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	13	0.0032371				
2	15	0.0068230	-2	-0.0035859	7.2003	0.007856 **

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Optimum temperature for growth

Analysis of Deviance Table

Model 1: T_opt ~ evolvedT

Model 2: T_opt ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)	
1	9	33.928				
2	11	71.641	-2	-37.714	5.0021	0.03461 *

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Net primary productivity at long-term growth temperature
Analysis of Deviance Table

Model 1: (NPP_cell) ~ evolvedT
Model 2: (NPP_cell) ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)	
1	13	0.014307				
2	15	0.030791	-2	-0.016484	7.489	0.00686 **

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Carbon-use efficiency at long-term growth temperature
Analysis of Deviance Table

Model 1: (CUE^2) ~ evolvedT
Model 2: (CUE^2) ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)	
1	12	0.04494				
2	14	0.65786	-2	-0.61292	81.841	1.016e-07 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Symbiont load at long-term growth temperature
Analysis of Deviance Table

Model 1: chlorella_per_um3_host_calc ~ evolvedT
Model 2: chlorella_per_um3_host_calc ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)	
1	13	7.6860e-09				
2	15	4.9834e-08	-2	-4.2148e-08	35.647	5.284e-06 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Maximum growth rate
Analysis of Deviance Table

Model 1: (mumax) ~ evolvedT
Model 2: (mumax) ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	14	0.0085396			
2	15	0.0090743	-1	-0.0005347	0.8766	0.365

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Grazing rate
Analysis of Deviance Table

Model 1: grazing_rate_day ~ Evolved_Temp
Model 2: grazing_rate_day ~ 1

Resid. Df	Resid. Dev	Df	Deviance	F	Pr(>F)
1	13	5.0214e+09			
2	15	5.4196e+11	-2	-5.3693e+11	695.04	6.09e-14 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Chapter 4: Table S2: Model selection for general linear models

Linear model selection tables for dependent variables as labelled. All global models are constructed as the dependent variable explained by long-term temperature. Null models were then constructed and were compared to the global model via ANOVA tests.

CUE at long-term growth temperature

Long-term temperature comparison

Temp (˚C)	diff	lwr	upr	p adj
25-20	-0.1185633	-0.2128184	-0.0243082	0.0146342
30-20	-0.5469417	-0.6623802	-0.4315032	0.0000001
30-25	-0.4283784	-0.5438169	-0.3129399	0.0000011

NPP at long-term growth temperature

Long-term temperature comparison

Temp (˚C)	diff	lwr	upr	p adj
25-20	-0.0007278893	-0.05130077	0.04984499	0.9992041
30-20	-0.0744858849	-0.13102808	-0.01794369	0.0106038
30-25	-0.0737579955	-0.13030019	-0.01721580	0.0112995

Symbiont load at long-term growth temperature

Long-term temperature comparison

Temp (˚C)	diff	lwr	upr	p adj
25-20	-3.641128e-05	-7.347773e-05	6.551697e-07	0.0543902
30-20	-1.310050e-04	-1.724466e-04	-8.956348e-05	0.0000039
30-25	-9.459375e-05	-1.360353e-04	-5.315220e-05	0.0001176

Optimum temperature for growth

Long-term temperature comparison

Temp (°C)	diff	lwr	upr	p adj
25-20	-0.4725692	-4.3057367	3.360598	0.9372241
30-20	3.5020414	-0.3311262	7.335209	0.0727709
30-25	3.9746105	0.1414429	7.807778	0.0425766

Symbiont growth on nitrate

Long-term temperature comparison

Temp (°C)	diff	lwr	upr	p adj
25-20	-0.006195894	-0.030251878	0.01786009	0.7789162
30-20	0.030915302	0.004019895	0.05781071	0.0242405
30-25	0.037111196	0.010215790	0.06400660	0.0077913

Symbiont growth on bacto-peptone

Long-term temperature comparison

Temp (°C)	diff	lwr	upr	p adj
25-20	0.09584397	0.03545908	0.15622885	0.0028249
30-20	-0.11000460	-0.17751695	-0.04249224	0.0023041
30-25	-0.20584857	-0.27336092	-0.13833621	0.0000058

Grazing rate at long-term growth temperature

Long-term temperature comparison

Temp (°C)	diff	lwr	upr	p adj
25-20	398636.62	368675.60	428597.64	0.0000000
30-20	57660.43	24162.99	91157.87	0.0014838
30-25	-340976.18	-374473.62	-307478.74	0.0000000

Chapter 4: Table S3: Tukey's multiple comparisons of means

Tukey's multiple comparisons of means tables displaying significant difference tests between variables as labelled at the three long-term temperature treatments (20°C ("cooled"), 25°C ("ambient/control") and 30°C ("warmed")).

(A) Symbiont density

Model selection table

	(Int)	evT	s(tmp,evT,6,F)	df	logLik	AICc	delta	weight
4	0.0001534	+	+	12	836.272	-1643.7	0.00	1
3	0.0001573		+	9	823.677	-1625.3	18.43	0
2	0.0001534	+		3	803.145	-1597.9	45.90	0
1	0.0001573			2	796.689	-1589.2	54.50	0

(B) Bacterivory rate

Model selection table

	(Int)	Evl_Tmp	s(Tmp,Evl_Tmp,4,F)	df	logLik	AICc	delta	weight
4	1614000	+	+	12	-1290.780	2611.8	0.00	1
3	1457000		+	8	-1405.683	2830.9	219.05	0
2	1614000	+		4	-1447.137	2902.7	290.90	0
1	1457000			2	-1462.535	2929.2	317.38	0

Chapter 4: Table S4: Model selection for generalised additive mixed effects models

Global models constructed to analyse the main effects of assay temperature (allowing for a non-linear relationship) and long-term temperature including the interaction effect between these two explanatory variables on a) symbiont density and b) bacterivory rate. Reduced models were constructed and all candidate models were ranked by AICc.

“This thesis is perfectly balanced, as all things should be.”

- Thanos