THE UNIVERSITY of EDINBURGH

Evolutionary consequences of viral resistance in the marine picoeukaryote *Ostreococcus tauri*

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Submitted for the degree of Doctor of Philosophy

At the University of Edinburgh

School of Biological Sciences

2017

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Signed Declaration

I declare that I have composed this thesis myself, that the work is my own, and that the thesis has not been submitted for any other degree or professional qualification. Below, I outline the contribution of my supervisors and colleagues to papers that were published based on data presented in Chapter 2 and Chapter 3 of this thesis.

The work presented in Chapter 2 was previously published in Environmental Microbiology. Sinéad Collins and I designed the experiment, analysed the data and wrote the manuscript. I performed the laboratory experiments.

A paper published in Viruses was based on data from Chapter 3. Sinéad Collins, Pedro F. Vale and I designed the experiments and analysed the data, Kirsten Knox and I performed the experiments, and all authors contributed to writing the manuscript.

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Abstract

In marine environments, eukaryotic marine microalgae coexist with the viruses that infect them. Marine microalgae are the main primary producers in the oceans and are at the base of the marine food web. Viruses play important roles in top-down control of algae populations, cycling of organic matter, and as evolutionary drivers of their hosts. Algae must adapt in response to the strong selection pressure that viruses impose for resistance to infection. In addition to biotic selection pressures such as viral infections, algae must also adapt to their abiotic environment. Global climate change is affecting temperature, salinity, pH, light and nutrient concentrations in the oceans, particularly in surface waters, where microalgae live. Currently, little is known about how consistent the effects of viruses on their hosts are, whether the cost of host resistance varies across environments, and whether there is a trade-off between maintaining resistance to viruses and adapting to other environmental changes.

The marine picoeukaryote *Ostreococcus tauri* is abundant in Mediterranean lagoons, where it experiences large fluctuations in environmental conditions and co-occurs with lytic viruses (*Ostreococcus tauri* viruses – OtVs). Viral infection causes lysis of susceptible (S) cells, however a small proportion of cells are resistant (R) and avoid lysis. Some resistant *O. tauri* populations can coexist with infectious viruses, and it has been proposed that these viruses are produced by a minority of susceptible cells within a mainly resistant population. These populations are referred to as resistant producers (RP). Virus production in RP populations is unstable and eventually they shift to R populations. I used *O. tauri* and one of its viruses, OtV5, as a model system to investigate whether cells that are susceptible or resistant to virus infection adapt to environmental change differently and whether there is a cost of being resistant.

For the first time, I evolved susceptible and resistant hosts of a marine alga separately under a range of environments and directly compared their plastic and evolved responses. I showed that resistant populations of *O. tauri* maintained their

resistance for more than 200 generations in the absence of viruses across all environments, indicating that the resistance mechanism is difficult to reverse. Furthermore, I did not detect a cost of being resistant, as measured by population growth rate and competitive ability. Virus production in RP populations stopped in all environments and all populations became R.

In addition, I found that virus production in RP *O. tauri* populations can fluctuate before completely ceasing, and that phosphate affected the length of time it took for virus production to stop. These results, combined with mathematical modelling of *O. tauri* infection dynamics, provide support for the prediction that RP populations consist of a mixed population of susceptible and resistant cells.

By examining multiple environments and resistance types, we can better understand first, how microalgae populations adapt to environmental change and second, the ecological and evolutionary consequences of maintaining resistance to viruses in common marine picoeukaryotes.

Lay Summary

The oceans are teeming with microorganisms. Tiny plant-like cells called microalgae are abundant at the surface of the oceans where they use sunlight to grow. Microalgae are important because they produce half of the oxygen we breathe and because they are at the base of the food web meaning all marine life depends on them to provide food.

When viruses attach to single-celled algae, they replicate within the cell before bursting it open to release all the newly made viruses. This causes the cell to die and in doing so releases all of its organic contents. This provides nutrients for other microorganisms that would not have otherwise been able to access them. So, viruses are important in recycling nutrients in the ocean. Not all algae are killed by viruses because some are able to evolve resistance. However, evolving resistance can come at a cost, and resistant cells can have a lower fitness (the ability to survive and reproduce) than susceptible cells when viruses are absent.

To survive, microalgae need to adapt to evolve resistance against virus infection, but at the same time they must also adapt to the physical environment in which they live and any changes that occur. Global climate change is causing changes in temperature, salinity, pH, light and nutrient concentrations. It is important to understand how environmental change will affect interactions between algae and viruses because this could affect global nutrient cycling, including carbon cycles, and impact marine communities and aquatic food webs.

To understand how resistance to viruses affects the ability of marine microalgae to cope with environmental change, I performed evolution experiments using *Ostreococcus tauri*, the smallest known green alga. *O. tauri* can be susceptible (S) to virus infection and die, or it can be resistant (R) and avoid infection. *O. tauri* can also coexist with viruses in populations that are thought to contain both S and R cells. These populations are called resistant producers (RP) because viruses are produced in the populations but overall the populations remain resistant. However,

virus production in RP populations is unstable and eventually all populations become R.

I grew populations of S, R and RP *O. tauri* separately in different environmental conditions for 200 generations to investigate if they evolved in different ways and to see if there was a cost of being resistant. I found that all population types were able to adapt to the new environments. Interestingly, R populations remained resistant, even though they were not evolving with viruses and therefore did not need to be resistant. Surprisingly, I also found that resistance was not costly for population growth rates or competitive ability in the laboratory environments that I used. All RP populations stopped producing viruses but remained resistant to new infections.

I performed further experiments that showed that the number of viruses in the RP populations fluctuated greatly before stopping completely, and that when *O. tauri* was fed less phosphate, virus production stopped more quickly. This laboratory experiment, along with mathematical modelling of the population infection dynamics, provided support for the hypothesis that RP populations are a mixture of both S and R cells, where the S cells eventually get overgrown by the R cells. Understanding more about virus infection in marine algae across many environments will help us understand the effects of climate change on microorganisms in the oceans. Here, I find that maintaining resistance to viral infection does not appear to limit adaptation to environmental change, at least within the conditions of laboratory experiments.

Acknowledgements

First, I wish to thank my supervisor, Sinead, for all of your help and encouragement. I am very grateful for your continuous enthusiasm and positivity which has motivated me during my PhD. Thank you to my second supervisor, Pedro, for your help and interesting discussions about modelling and experimental design. This leads me to the rest of my lab group, Rasmus, Heidi, Elisa, Georgina and Diane, for providing the perfect balance of technical support and trips to the Chocolate Tree. Thank you Rasmus, for helping me with lab work and for discussions about evolutionary biology. Thank you Elisa, for replying to every single one of my emails and for always making time to see me when you were in Edinburgh. I also thank Nikola, Josianne and others from the algae lab groups for your support over the last few years. Thank you to all the office mates for always providing a fun and friendly environment to work in and an endless supply of cake. This project was made possible with support from an EASTBIO DTP funded by the BBSRC.

I wish to thank everyone in the lab at Banyuls for teaching me how to work with marine viruses and for sending me lots of algae strains. I also thank everyone in the Millar lab for advice working with *Ostreococcus*, valuable help with experiments and modelling, and friendship. Thank you to Martin Waterfall for letting me loose on the flow cytometer and Luke McNally for advice with epidemiological modelling.

I am grateful for the support of my friends. Thanks for the hiking trips, badminton, coke breaks, drinks at the Old Bell and Summerhall, Meadows barbeques, roast dinners, tiramisu, flat mate chats and gentlemen outings. Thanks Tom for the time spent helping me with my PhD and even more time spent escaping the lab for new adventures. My friends from London, Liverpool and Plymouth were only a phone call away and provided many weekends of laughs and breaks from the PhD life. Thanks for always being there for me despite the hundreds of miles between us.

Finally, I thank my parents for your endless love and support. You gave me the opportunity to follow my heart, for which I am truly grateful.

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1. Introduction

Viruses were first discovered only one hundred years ago. In 1915, Frederick Twort accurately described the action of viruses that infected bacteria, and in 1917, Félix d'Herelle independently observed bacterial lysis resulting from virus infection and named the causative agents "phage" (which in Latin literally means "to devour"). Viruses are obligate parasites meaning they can only reproduce upon infection of a host cell. For this reason, biologists often do not consider viruses to be "alive" and the debate is still ongoing (Moreira and López-García, 2009). However, viruses undoubtedly possess characteristics of life, such as nucleic acids, genes, and the ability to evolve and adapt to infect specific hosts. Thus, whether viruses are alive is irrelevant when observing them from an eco-evolutionary context since they are hugely important in shaping the evolution of their hosts by imposing strong selection pressures to avoid viral infection.

Viruses are the most abundant biological entities on the Earth. While most attention is usually focused on the few viruses that cause serious human diseases, the majority of the world's viruses are found in the oceans. There are an estimated 10^{30} virus-like particles in the oceans, most of which infect microorganisms (Suttle 2005). The field of marine virology has expanded over the past thirty years, from initially focusing on viral abundance (Bergh *et al.*, 1989), to incorporating these data into the microbial loop (Fuhrman, 1999; Wilhelm and Suttle, 1999). Currently, much attention is focused on sequencing metagenomes from global ocean samples to quantify viral diversity (Sharon *et al.*, 2011; Parsons *et al.*, 2012; Hurwitz and Sullivan, 2013). The numbers of viruses in the oceans can be up to orders of magnitude higher than microbial cell abundance, depending on the region (e.g. the oligotrophic open ocean, nutrient rich coastal waters, sediments, abyssal depths, tropical or polar regions) (Fuhrman, 1999). It was recently revealed that there is substantial variation between near-surface and sub-surface water and the virus-to-microbe cell ratio is not linear, typically decreasing with microbial cell density (Wigington *et al.*, 2016). However, the relationship between marine viruses and hosts varies between studies (Knowles *et al.*, 2016; Wigington *et al.*, 2016). Due to their high density, viruses have profound

effects on the cellular hosts they encounter. This is particularly true for unicellular marine microbes, such as bacteria and eukaryotic protists, since viruses are a major source of mortality.

Marine phytoplankton are important primary producers at the base of the food web and must adapt not only to avoid viral infection but also to the abiotic environment in which they live. Most of the anthropogenic CO_2 released into the atmosphere is absorbed into the oceans (Caldeira and Wickett, 2003) causing changes in pH, temperature and solar radiation (IPCC Fifth Assessment Report 2014). This can influence ocean currents, which in turn can change nutrient distributions and salinity. These changes are happening on a global scale, but marine organisms also face local environmental changes and gradients. For example, lagoons and coastal waters can experience large daily fluctuations in salinity, temperature and nutrients due to evaporation, rainfall, and water exchange with rivers (Bellec *et al.*, 2010).

In this chapter, I will limit descriptions of virus infection to cases where the hosts are unicellular organisms, since my thesis focuses on a unicellular green alga. A virus infects a host cell by attaching to specific cell surface receptors, usually proteins that already serve other functions for the cell (e.g. nutrient uptake). Once attached, the virus injects its genetic material into the cell. It then hijacks the cell's replication machinery to replicate its own DNA or RNA, and new virions are assembled. Eventually the host cell ruptures to release the newly made virions (Brussaard, 2004). This rupturing is referred to as lysis and inevitably results in mortality of single cells.

In my thesis, I focus on the evolutionary implications of maintaining host resistance in the single celled marine alga *Ostreococcus tauri*. In the past decade, *O. tauri* has been intensively studied and is now recognised as a model organism. In this introductory chapter, I will outline the important roles that marine viruses play in the oceans, and then explore the importance of viruses and host resistance in the field of evolutionary biology and how these roles could be affected by environmental change.

1.1 The roles of marine viruses & phycodnaviruses in the oceans

Marine viruses play many important roles in the oceans. They are responsible for a large portion of cell death of marine microorganisms, they directly influence biogeochemical cycles, and they shape the growth and evolution of their hosts.

1.1.1 Ecological roles of marine viruses

Viruses may be the smallest "life forms", but they are also the most abundant. Although they comprise only 5% of the total biomass, they account for 94% of the nucleic acid containing particles in the oceans (Suttle, 2007). Approximately 10^{23} viral infections occur every second (Suttle 2007), making viruses a major cause of mortality to marine organisms which influences population abundances and community dynamics at all trophic levels. Additionally, the death of unicellular organisms through viral lysis mediates the movement of organic material from living organisms to particulate and dissolved organic matter via a process termed the viral shunt (Wilhelm and Suttle, 1999). This shunting alters carbon and nutrient cycling and makes organic matter available to organisms at lower trophic levels.

Viral attacks can also play a role in species succession during phytoplankton blooms (Schroeder *et al.*, 2003), adding complexity to microbial community composition in the oceans. For instance, blooms formed by the coccolithophore *Emiliania huxleyi* are initially driven by increased nutrients, but can crash earlier and more rapidly than would otherwise be expected due to viral attack because the high host cell density leads to a high virus-host encounter rate (Brussaard, 2004).

1.1.2 Evolutionary roles of marine viruses

Many ecological studies performed on marine viruses have focused on their importance in cell mortality, and it is now widely accepted that they play a huge role in energy and nutrient cycling. Although viruses are the most abundant biological entities on the planet, less is understood about their evolution than is understood for

cellular organisms, particularly because viruses do not share any universal genes (Iranzo *et al.*, 2016). We know that viruses can be key drivers of host cell evolution through coevolution, competition, maintaining diversity and horizontal transfer (i.e. the direct acquisition) of genetic material, however most of this evidence comes from bacteria-phage studies (Koskella and Brockhurst, 2014). Here, I will provide some examples of marine viruses imposing large selective pressures on their hosts and thereby driving phytoplankton evolution.

The strongest selection pressure that viruses exert on their hosts is for resistance against infection. Antagonistic coevolution is the reciprocal evolution of host resistance and virus infectivity (Buckling and Rainey, 2002). Two types of dynamics driven by antagonistic coevolution are arms race dynamics and fluctuating selection dynamics. Viruses and hosts can evolve in a coevolutionary arms race, where both are constantly adapting to outcompete each other. Directional selection occurs when hosts are adapting to gain broader resistance from viruses in parallel to the viruses evolving to overcome the host's defence mechanisms (Buckling and Rainey, 2002). This favours hosts that are resistant to many viruses and viruses that can infect many hosts. This was shown in chemostat experiments when coevolution between hosts and their viruses evolved increased host resistance and increased virus infectivity in marine cyanobacteria (Marston *et al.*, 2012) and in the freshwater alga *Chlorella variabilis* and its lytic virus PBCV-1 (Frickel *et al.*, 2016). In contrast to directional selection, fluctuating selection occurs when different host and virus genotypes fluctuate in frequency over time, as they evolve different rather than increased resistance and infectivity ranges, respectively (Buckling and Rainey, 2002; Avrani *et al.*, 2012). As the interacting species adapt in response to each other, their average fitness remains constant (Brockhurst *et al.*, 2014). This could explain the long-term genotypic stability and apparent stable community observed in the marine coccolithophore *Emiliania huxleyi* and its viruses (Martinez Martinez *et al.*, 2007). Antagonistic coevolution is likely important in generating and maintaining genetic and phenotypic diversity (Martiny *et al.*, 2014), probably mainly through trade-offs that result in hosts occupying different ecological niches (Brockhurst *et al.*, 2004; Frickel *et al.*, 2016). One study examining the effect of spatial heterogeneity on host

diversity in the bacterium *Pseudomonas fluorescens* found that diversity was increased under resource competition due to trade-offs between resistance and competitive ability (Brockhurst *et al.*, 2004). Selection for multiple modes of resistance, through underlying genotypes or different phenotypes, also increases diversity (Brockhurst *et al.*, 2004).

The dynamics of host-virus evolution have important ecological consequences in terms of shaping host populations. For example, the "Kill the Winner" hypothesis proposes that the population of phytoplankton that becomes most abundant, for example the fastest growing genotype, consequently becomes the target for virus infection (Thingstad, 2000). This is particularly relevant to algal blooms, which are often terminated by viral lysis (Bratbak *et al.*, 1993; Jacquet *et al.*, 2002). Kill the winner strategies can also lead to increased diversity over time as different genotypes take it in turns to dominate.

Although coevolution has been studied extensively in a wide variety of organisms (Brockhurst *et al.*, 2014), very few studies have considered eukaryotic algae. Any environmental change that affects host-virus coevolution could have important consequences for marine ecology (Northfield and Ives, 2013). Thus, there is a large need for a better understanding of algae-virus interactions to allow us both to understand the role of primary producers in aquatic systems, and to predict the evolutionary responses of marine microorganisms to environmental change in the oceans.

1.2 Marine eukaryotic algae and the picoeukaryote *Ostreococcus tauri*

Marine phytoplankton play a central role in marine biogeochemical cycles and food webs. Despite their microscopic size, they are responsible for as much as half of the global primary production (Field, 1998) and are at the base of marine food webs (Falkowski, 1998a). Climate change could cause changes to phytoplankton communities, size structure, population abundance or spatial range. This could alter or generate novel ecosystems by promoting migration or extinction if phytoplankton

cannot adapt to their new conditions (Doney *et al.*, 2012). Large changes in populations could modify nutrient cycles (including carbon) and thus eventually the ecosystem functioning upon which all species depend.

Picophytoplankton comprise both prokaryotic and eukaryotic cells <2 µm in size (Worden *et al.*, 2004). Photosynthetic picoeukaryotes, although not numerically dominant, are important primary producers and can be responsible for three quarters of the picoplankton primary production and nearly 80% of the carbon consumption by higher trophic levels in oligotrophic regions (Worden *et al.*, 2004). Their tiny size and large surface area to volume ratio gives picophytoplankton a competitive advantage over larger cells in oceanic regions with lower nutrient concentrations (Falkowski, 1998b).

The picoeukaryote *Ostreococcus tauri*, first isolated from the Thau lagoon (Courties *et al.*, 1994), belongs to the order Mamiellales at the base of the green lineage (Derelle *et al.*, 2006). It is the smallest eukaryote described to date with a diameter of 1 µm, and has a very simple structure containing only one mitochondrion and one chloroplast and lacking a cell wall. *O. tauri* has a compact 13 Mb haploid genome which has a high level of heterogeneity and two atypical "outlier" chromosomes (Derelle *et al.*, 2006; Blanc-Mathieu *et al.*, 2014), which may be involved in virus resistance (Yau *et al.*, 2016) (discussed in section 1.3.2). These two chromosomes, 2 and 19, differ structurally from the other 18 by having a higher GC content and containing three quarters of the transposable elements (Derelle 2006). Chromosome 19 is particularly unusual in that its genes share no phylogenetic relationship with the green lineage and many are weakly related to bacterial proteins, leading to the suggestion that this entire chromosome was horizontally transferred to *O. tauri* from another organism.

O. tauri is widely distributed (Worden *et al.*, 2004; Countway and Caron, 2006), but the most studied strains come from the Mediterranean, where it is the dominant alga in lagoons (Viprey *et al.*, 2008). Due to its primitive cell structure, available genome sequence and ease of culturing, *O. tauri* is now widely used as a model organism for

studies on the cell cycle (e.g. Robbens *et al.* 2005, Farinas *et al.* 2006), circadian rhythms (e.g. Moulager *et al.* 2007, Corellou *et al.* 2009), proteomics (Le Bihan *et al.*, 2011), climate change (Schaum *et al.*, 2012, 2015; Schaum and Collins, 2014) and host-virus interactions (e.g. Bellec *et al.* 2010, Thomas *et al.* 2011, Clerissi *et al.* 2014). I take advantage of these features and use it as a model for experimental evolution studies of environmental change and virus infection. *O. tauri* reproduces asexually and typically divides once per day (Moulager *et al.*, 2007). This short generation time means that *O. tauri* populations will experience climate change more gradually relative to slower growing organisms, potentially allowing them more opportunity to adapt to change.

1.3 *Ostreococcus tauri* viruses (OtVs) and host virus resistance

1.3.1 OtVs are abundant in their host's habitats

The first report of observations of virus-like particles in *O. tauri* came from electron micrographs of samples taken from a transient bloom near Long Island in 2003 (O'Kelly *et al.*, 2003). Since then, three *O. tauri* viruses, better known as OtVs, have had their genomes sequenced and they are all reported to be lytic viruses (Derelle *et al.*, 2008; Weynberg *et al.*, 2009, 2011). OtVs are prasinoviruses belonging to the *Phycodnaviridae* family. Although the *Phycodnaviridae* are genetically diverse, they are morphologically similar and fall within the nucleo-cytoplasmic large double stranded DNA viruses (NCLDVs) group (Iyer *et al.*, 2001, 2006). OtVs have small linear genomes ranging from 184 to 192 kbp. Their capsids are approximately 100-120 nm in length, making them huge compared to their 1 μm hosts. Virus replication takes place in the cytoplasm, and since the *O. tauri* cell is very compact (Henderson *et al.*, 2007), the burst size is only about 25 viruses per cell (Derelle *et al.*, 2008). In contrast, the picoeukaryote *Micromonas pusilla* has an estimated burst size of 500 viruses per cell and most other algae have burst sizes of thousands (Short, 2012), making the *O. tauri*/OtV system a unique one.

OtVs are frequently detected in water samples collected from lagoons and coastal waters (Bellec *et al.*, 2009; Bellec, Grimsley, Derelle, *et al.*, 2010), showing that they

are abundant in the habitat of their *O. tauri* host (Derelle *et al.*, 2008). Most OtVs have strict host strain specificity, while others can infect several hosts (Derelle *et al.*, 2008; Clerissi *et al.*, 2012; Bellec *et al.*, 2014).

1.3.2 OtV infection and O. tauri resistance

Virus infection of *O. tauri* usually causes cell lysis of susceptible (S) cells (Derelle *et al.*, 2008). However, resistance always arises in response to OtVs in the laboratory (Thomas *et al.*, 2011). Interestingly, following growth of these resistant cells, the population remains resistant upon re-infection (Thomas *et al.*, 2011). Experimental evidence indicates that resistance to OtVs is an intracellular response that is gained through structural rearrangement of the outlier chromosome 19 (Yau *et al.*, 2016). Both genetic and epigenetic changes are likely to be involved, and the low chance of reversing all of these changes probably explains why these cells retain their resistance, even hundreds of generations after viruses have been removed from the population (Thomas *et al.*, 2011; Yau *et al.*, 2016; Heath *et al.*, 2017). I discuss the maintenance of resistance further in Chapter 3.

Two types of resistant populations have been observed in the laboratory (Thomas *et al.*, 2011; Yau *et al.*, 2016). In the first type, viruses are able to attach to the cell surface but they are unable to infect the cell and cause cell lysis. These cells are referred to as resistant (R). In the second type, populations as a whole are resistant to lysis upon virus infection, however infectious virus particles are present in the cultures. In Chapter 4, I show that viruses can be detected in these cultures over several transfers, indicating they are being actively produced over many host generations. These populations are referred to as resistant producers (RP) because of the viral production in the cultures. Throughout this thesis, I refer to the three cell types (S, R and RP) as resistance types.

When the R and RP resistance types were first identified, it was originally thought that *O. tauri* had two distinct resistance strategies. Thomas *et al.* (2011) proposed that cells in the RP populations were chronically infected by viruses, and that instead

of lysing, they slowly released the viruses via budding. However, after re-examination of electron micrographs of the RP cultures, individual cells were observed to be undergoing cell lysis (Yau *et al.*, 2016). Thus, a new hypothesis was proposed that in fact RP cultures consist of a majority of resistant (R) cells and a minority (<0.5%) of susceptible (S) cells that become infected and thus maintain a population of infectious viruses. Interestingly, RP populations do not appear to be stable and in a study in Chapter 3 (Heath *et al.*, 2017) and a separate study by Yau *et al.* (2016) it was found that eventually viruses are no longer detected in RP populations and that these populations eventually evolve to consist solely of R cells.

In this thesis, I use different genotypes of S, R and RP *O. tauri*, which I refer to as lines. These lines were first isolated by N Grimsley and have been maintained in the laboratory for several years as follows. There were two starting dates from which the cultures were first isolated; the first and second series are named NGxx and NG'xx, respectively. The original clone for NGxx was made and grown up in April 2013. Following virus treatments, regrowth and re-plating, resistant clones were obtained in August 2013. The original clone for NG'xx was from the same cells as the NGxx which was revived from a frozen stock. These were plated for new single colonies that grew up and were infected and re-plated in January 2014 to obtain resistant clones. Susceptible clones were made in parallel alongside the resistant clones. Populations of S, R and RP *O. tauri* were maintained at Ashworth Laboratories, University of Edinburgh, since January 2015 in Keller media by serial transfer.

1.4 Resistance to viruses and fitness trade-offs – does resistance come at a cost?

Various strategies for virus resistance have been reported in microalgae, including changes to cell surface receptor proteins (Tarutani *et al.*, 2006), activation of programmed cell death (Bidle *et al.*, 2007), absence of metacaspase (caspase orthologues) protein expression (Bidle *et al.*, 2007), stage of the life cycle (Frada *et al.*, 2008), colony formation (Brussaard *et al.*, 2007), genetic mutations (Stoddard *et al.*, 2007) and chromosomal restructuring (Yau *et al.*, 2016).

If single celled marine algae are able to acquire resistance to viruses relatively quickly due to their rapid generation times and large population sizes, why do resistant cells not take over the population and out-compete susceptible cells? Evolutionary theory predicts that this paradox must be explained by a fitness trade-off to being resistant to viral lysis, or a so-called cost of resistance, in order for both susceptible and resistant cells to coexist. Associated costs with resistance to viral lysis have important evolutionary consequences, as they will affect the population dynamics and community structure of hosts and viruses. A trade-off can be described as a characteristic which is beneficial in one circumstance but deleterious in another. Trade-offs are one part of explanations for biodiversity and how multiple strains or species are able to coexist in an environment.

When a mutation conferring resistance arises, it is likely to cause changes to the original cell physiology, thereby reducing fitness if there is full or partial loss of the normal function where the mutation occurred (Lenski, 1998). Consequently, in the presence of viruses, the resistance mutation will provide a fitness advantage to the cell compared to susceptible cells. However, when viruses are removed from the environment, if the cost of the resistance mutation is large, the resistant cells will have a lower fitness than their susceptible counterparts, and resistance should be selected against and lost. It is logical to predict that in order for susceptible cells to persist in an environment, there must be a cost of being resistant, otherwise all cells would be resistant. A cost of resistance could be the result of antagonistic pleiotropy, when an allele that has a fitness advantage in one environment is disadvantageous in another environment, or mutation accumulation, when mutations are accumulated in one environment that are detrimental in a different environment (Elena and Lenski, 2003).

Resistance to viral lysis can come at different pleiotropic fitness costs. Reduced growth rate is often speculated to be a cost of resistance however it has rarely been observed. Where it has been observed, for example in the ubiquitous cyanobacteria *Synechococcus* (Lennon *et al.*, 2007), and *Prochlorococcus* (Avrani *et al.*, 2011) a

growth cost was only reported in approximately half of the strains tested. Alternatively, resistance to one virus can lead to increased susceptibility to infection by other viruses (Avrani *et al.*, 2011). This enhanced infection might occur in *Ostreococcus*, whose viruses are mainly species specific (Clerissi *et al.*, 2012). Avrani and Lindell (2015) suggest that independent resistance of phytoplankton (in this case the cyanobacterium *Prochlorococcus*) to multiple phages can increase genetic diversity and enable resistant cells in nature to grow close to their maximum growth rates.

Although it is widely assumed, evidence for a cost of resistance is sparse. This is especially true for eukaryotes, since the majority of experiments examining cost of resistance in microorganisms have used *E. coli* (Bohannan and Lenski, 2000). Interestingly, one reason for the lack of information on a cost of resistance is that in the absence of the virus the cost can rapidly be reduced or even lost completely. This can either be because of compensatory mutations (Lenski, 1988b), or because resistant cells with the lowest fitness cost will be strongly selected for (Lenski, 1988a). Another reason why costs of resistance can often be difficult to detect is that under rich laboratory conditions, the environment is often set at the optimum conditions for growth (e.g. in terms of nutrients, light, temperature). A cost might therefore be absent or below the detection limit with the available technology (Lennon *et al.*, 2007), whereas in the natural environment, a cost could be more pronounced.

Thus, the question still remains: does a cost of resistance in marine algae really exist? There is a high abundance of viruses in marine ecosystems (Suttle, 2005) and some researchers have predicted that almost all marine bacteria are sensitive to phage (Fuhrman, 1999; Wommack and Colwell, 2000), although less is known about eukaryotic microbes. Since we know hosts can rapidly gain resistance to their viruses, this is intriguing, and a cost of resistance could provide an explanation for this paradox.

1.5 Environmental change and effects on algal hosts and viruses

Environment can have two main effects on virus abundance and productivity. First, there can be a *direct* effect, in which a virus particle could be damaged or inactivated due to a change in the surrounding conditions. Examples include high temperature and UV exposure (Jacquet and Bratbak, 2003; Wells and Deming, 2006). Alternatively, since viruses are completely reliant on their hosts for replication, when host abundance is altered, for example in response to environmental changes, this will have an *indirect* impact on virus abundance, by altering encounter rate depending if there is an increase or decrease in available cells to infect (Danovaro *et al.*, 2011).

O. tauri naturally inhabits Mediterranean lagoons that are connected to the open ocean via narrow channels (Bellec, Grimsley, Derelle, *et al.*, 2010). These channels limit the exchange of seawater between the lagoon and ocean, making variations in factors such as salinity, pH, temperature and nutrients more extreme. *O. tauri* and OtVs must be adapted to cope with the selective pressures of these large fluctuations.

If species have strong coevolutionary relationships, as is the case in host-virus interactions, environmental change that affects one of these species will consequently indirectly affect the other. This could disrupt the balance between normal ecosystem functioning (Northfield and Ives, 2013). Models have shown that coevolution can reduce the effects of climate change if species have conflicting interests, and vice versa (Northfield and Ives, 2013). Increasing nutrient concentrations increased bacteria productivity and also the rate of coevolution between the bacteria and phage, probably because of increased encounter rates with the higher bacteria density (Lopez-Pascua & Buckling 2008). These findings show that we must not only investigate the impacts of climate change on single species, but on communities, since changes in one species will have knock-on effects.

1.6 Evolution in action - microbes are widely used in experimental evolution

Experimental evolution is the study of evolution in real time. Microorganisms are the perfect candidates for laboratory evolution experiments since they reproduce (divide) rapidly (from hours to days), allowing hundreds of generations and large populations to be grown over relatively short time scales (Adams and Rosenzweig, 2014). Additionally, environmental variables can be manipulated relatively easily, experiments using large populations can be replicated many times, and many microbial species can be stored in suspended animation to allow the direct comparison of ancestors and evolved lines (Elena and Lenski, 2003). The basis for microbial experiments is often simple in that populations are established from single clones and then evolved in a reproducible environment for many generations with replicates (Elena and Lenski, 2003). Adaptation to the novel environment is measured by comparing fitness of the evolved population relative to that of its ancestor or evolving control populations in that environment. In my thesis, I use *O. tauri* as a tool for experimental evolution to investigate host resistance to viruses and how this could be affected by environmental change. Since *O. tauri* is an ecologically important species, these results can be used to shed light on the effects of climate change on marine eukaryotic algae host-virus interactions.

1.7 Evolutionary and ecological consequences of host resistance

It is important to understand how interactions between marine microbes can be affected by climate change, because it could have large effects on nutrient cycling, community composition and population dynamics. Understanding the relationship between microbes and their environments is also a necessary part of increasing our fundamental understanding of how ecosystems function. Without experiments and field studies, we cannot predict how virus infection and host resistance might change with a changing environment. By examining many environments and by directly comparing sensitive and resistant populations, I can disentangle whether responses of *O. tauri* lines to environmental changes are likely to differ based on their resistance types and if so by how much. In addition, to understand the implications of host

resistance, we must find out what drives cells to become susceptible or resistant to viruses. *O. tauri*-OtV5 provides a unique and interesting study system to understand how resistance and/or tolerance to viruses evolves since little work has focused on virus infection in marine eukaryotic algae. The unique RP strategy, that has so far only been reported in *O. tauri*, also raises questions as to the ecological effects of the coevolutionary dynamics of this system.

1.8 Thesis overview

I use experimental evolution and the model organism *Ostreococcus tauri* to examine the effect of virus resistance on adaptability to environmental change and the selection pressure for resistance.

First, I compare the plastic and evolutionary responses of different *O. tauri* resistance types to different environmental changes. I show that resistant producer (RP) populations of *O. tauri* can have a growth advantage across a range of environments, except in an environment where they grow equally as badly as susceptible and resistant lines (Chapter 2). I also show that resistance to viruses is maintained both immediately after exposure to a novel environment and after evolution in that environment. Using several measures of fitness, I demonstrate that resistance is not costly in *O. tauri* in a laboratory setting (Chapter 3). In addition, I find that RP lines stop producing viruses under all environmental conditions.

Second, in light of results from the first two chapters, I examine further the population dynamics of RP *O. tauri* lines. I use laboratory experiments to show that virus production fluctuates in RP populations before stopping completely, and that phosphate concentration can limit the number of viruses produced (Chapter 4). Finally, I use modelling to provide support for the hypothesis that RP populations consist of both susceptible and resistant cells (Chapter 5).

2. Mode of resistance to viral lysis affects host growth across multiple environments in the marine picoeukaryote *Ostreococcus tauri*

2.1 Abstract

Viruses play important roles in population dynamics and as drivers of evolution in single-celled marine phytoplankton. Viral infection of *Ostreococcus tauri* often causes cell lysis, but two spontaneously arising resistance mechanisms occur: resistant populations that do not lyse and resistant producer populations that do not lyse but maintain infectious viruses within the culture. As of yet, little is known about how consistent the effects of viruses on their hosts are across different environments. To measure the effect of host resistance on host growth, and to determine whether this effect is environmentally dependent, I compared the growth and survival of susceptible, resistant and resistant producer *O. tauri* cells under five environmental conditions with and without exposure to *O. tauri* virus. While the effects of exposure to virus on growth rates did not show a consistent pattern in populations of resistant cells, there were several cases where exposure to virus affected growth in resistant hosts, sometimes positively. In the absence of virus, there was no detectable cost of resistance in any environment, as measured by growth rate, cell size and cell chlorophyll content. In fact, the opposite was the case, with populations of resistant producer cells having the highest growth rates across four of the five environments.

2.2 Introduction

Marine viruses play a large role in nutrient and energy cycling in the oceans. Viral lysis of single celled organisms releases large quantities of organic matter into the environment, making nutrients available for use by bacteria and algae. This process has been termed the viral shunt (Wilhelm and Suttle, 1999). Studies on marine viruses typically focus on the importance of viruses in nutrient cycling and the release of organic matter through cell lysis. Despite the important role of marine viruses in ecosystem function across many environments, from nutrient rich coastal waters to more oligotrophic regions of the open ocean (Brussaard, 2004), host–virus interactions are typically studied in single environments. Here, I use the *Ostreococcus tauri/Ostreococcus tauri* virus model system to investigate variation in host–virus interactions across environments to understand (1) whether susceptibility/resistance to viruses changes with environmental change and (2) whether the growth effect of host resistance depends on environmental context or resistance type.

I explore the relationship between host responses to environmental change and the resistance strategies of those hosts using the marine picoeukaryote *Ostreococcus tauri* (order Mamiellales). *O. tauri* is commonly isolated from Mediterranean lagoons that are connected to the open ocean via narrow channels (Clerissi, Grimsley, Subirana, *et al.*, 2014). These channels limit the exchange of seawater between the lagoon and ocean, making variations in the environmental salinity, pH, temperature and nutrients more extreme than in the open ocean (Bellec, Grimsley, Derelle, *et al.*, 2010; Clerissi, Grimsley, Subirana, *et al.*, 2014). *Ostreococcus tauri* viruses (OtVs) have been sampled frequently in seawater collected from lagoon and coastal waters where *O. tauri* is found. OtVs have strict host specificity (Clerissi *et al.*, 2012), and the three OtVs sequenced to date have all been described as lytic viruses (Derelle *et al.*, 2008; Weynberg *et al.*, 2009, 2011). Virus infection of *O. tauri* usually causes cell lysis in susceptible (S) cells, though two mechanisms of resistance have been observed (Thomas *et al.*, 2011). In the first case, viruses are unable to infect and lyse the host, and these cells are referred to here as resistant (R).

In the second case, it is currently hypothesised that hosts are mainly resistant to lysis but coexist with a small proportion of susceptible cells that maintain a virus population through infection and lysis (Yau *et al.*, 2016). These cells are termed resistant producers (RP).

Resistance type could have consequences for growth and other cell properties, such as size and chlorophyll content. For example, a trade-off of acquiring resistance to viral lysis may come as a fitness cost. This often occurs as reduced competitive ability (Lenski, 1988a; Bohannan *et al.*, 2002) and sometimes reduced growth rate (Lennon *et al.*, 2007; Frickel *et al.*, 2016). A modification in cell surface receptors to limit virus attachment could also result in a loss of the original function of the protein, such as metabolism or being able to target the host immune system. In several bacteria species, loss of a bacteriophage receptor results in lower virulence of the bacteria in its host, thereby lowering the fitness of resistant compared to non-resistant strains (Seed *et al.*, 2012; León and Bastías, 2015). Lastly, strong resistance to one specific virus strain may lead to increased susceptibility to lysis by other strains, as has been observed in *O. tauri* (Clerissi *et al.*, 2012) and cyanobacteria (Marston *et al.*, 2012; Avrani and Lindell, 2015).

The group of viruses that infects some eukaryotic algae is the Phycodnaviruses. These viruses have been studied under environmental conditions that differ from a benign control environment in a single driver, such as increases in temperature (Nagasaki and Yamaguchi, 1998; Wells and Deming, 2006), nutrient (Bratbak *et al.*, 1993, 1998; Bellec *et al.*, 2010; Clerissi, Grimsley, Subirana, *et al.*, 2014), light (Bratbak *et al.*, 1998; Weinbauer, 2004), UV (Jacquet and Bratbak, 2003), CO_2 (Larsen, a. Larsen, *et al.*, 2007; Chen *et al.*, 2014; Maat *et al.*, 2014) and pH levels (Weinbauer, 2004). When environmental conditions are stressful, one consequence can be inactivation of the virus particle. This affects host–virus interactions by preventing infection through structural degradation, the inability of the virus to inject its genome into the host or the inability of the virus to replicate (Børsheim, 1993; Jacquet and Bratbak, 2003). Additionally, since viral replication and life cycle are often closely linked to host metabolism, environmental changes such as increased

temperature or nutrients will often have an indirect effect on responses to viral attack (Weinbauer, 2004; Danovaro *et al.*, 2011). Understanding the role of viruses in marine communities requires investigating their activity across environments. Here, I focus on the environmental changes of increased temperature, decreased nutrients, decreased light and decreased salinity levels.

Previous studies of resistance in *O. tauri* found that when each resistance type was maintained separately there was no significant difference in growth rates, such that a cost of resistance was too low to be detected by differences in growth alone. However, when resistant types were competed against each other, a competitive hierarchy was observed in which S had the fastest growth rate, followed by R and then by RP (Thomas *et al.*, 2011). Since the three resistance types share the same starting genotype, it is possible to make direct comparisons between them. In this study, I performed an experiment in which three populations of each *O. tauri* resistance type (S, R and RP) derived from a common ancestor were grown for one week in the following environments in the absence of OtV5 virus: high temperature, low light, low phosphate and low salt. These environments were selected to represent relatively small variations from the control environment in which the populations are normally maintained in the laboratory, so that the cells responded, but were still able to grow at a rate that was measureable. The average number of cell divisions per day over a single transfer cycle (7 days), cell size and cell chlorophyll content were measured in the novel environments in the absence of OtV5. Offspring production over a fixed period of time is a proxy for fitness in single celled organisms in batch culture experiments (Brennan and Collins, 2015). Cell size and chlorophyll content were measured as additional phenotypes, to examine effects on organismal function other than cell division rates, since only small differences in growth were detected previously (Thomas *et al.*, 2011). After one week of growth in the novel environment, all populations were inoculated with OtV5 and cell densities were measured three days after inoculation to test for susceptibility to viral lysis.

2.3 Methods

2.3.1 Susceptible and resistant lines used in this experiment

O. tauri populations were obtained from N. Grimsley, Observatoire Oceanologique, Banyuls-sur-Mer. Three susceptible (S) lines (NG'2, NG'3 and NG'4), three resistant producer (RP) lines (NG'10, NG'16 and NG27) and three resistant (R) lines (NG5, NG'13 and NG26) were used in this study. I used three biological replicates for each line in each environment, which I refer to as populations. All lines were derived from a single clone of *O. tauri* (RCC 4221) and therefore had the same starting genotype (see Thomas *et al.*, 2011). All populations have since been maintained separately.

All RP populations were tested for viral production prior to the start of the experiment. To do this, I used the supernatant of these strains to infect susceptible *O. tauri* cells. Populations NG'10, NG'16 and NG'27 were aliquoted into 2ml Eppendorf tubes and centrifuged at 4000 × g for 15 min. Next, 400 μl of supernatant was carefully removed without drawing up any cells from the pellet at the bottom of the tube, and used to inoculate 1 ml of susceptible *O. tauri* strain RCC4221. Eight replicates were performed. A positive control was performed using known OtV5, and a negative control was performed by adding Keller media. Controls were performed in quadruplicate. Cells were left to grow for 3 days after which their densities were measured using a FACSCanto flow cytometer. I observed cell lysis resulting from inoculation with supernatant from all three RP populations, showing that there was active virus in the media taken from these cultures (Figure 2.1).
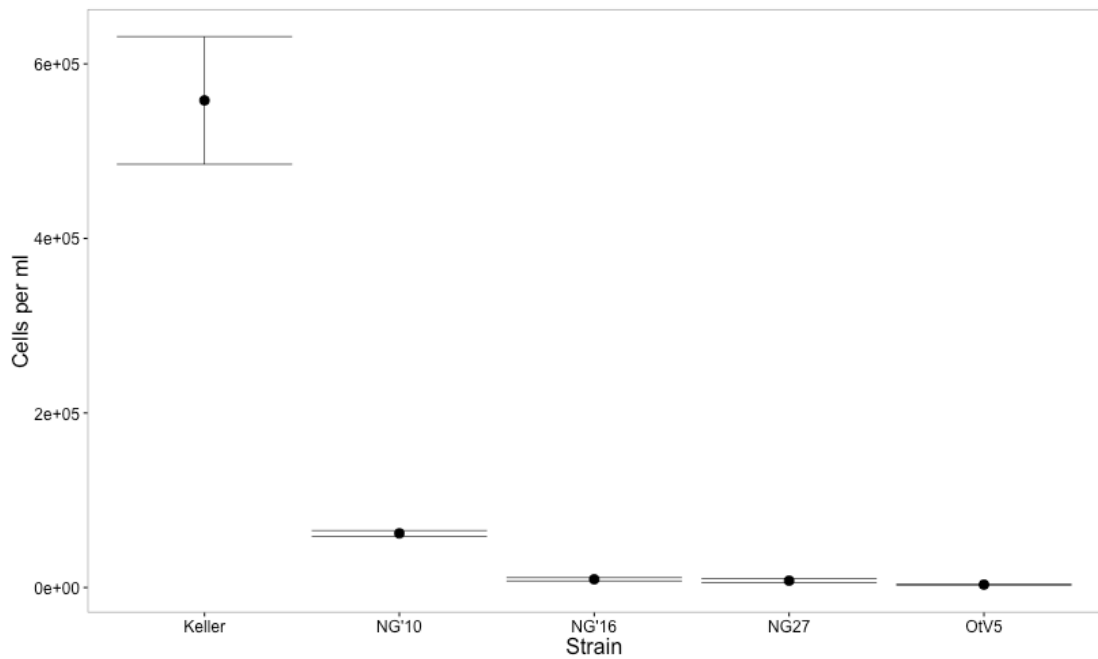
Figure 2.1. Mean cell densities ml-1 (±SEM) of *O. tauri* strain RCC4221 three days after inoculation with supernatant from three resistant producer populations (NG'10, NG'16 and NG27).

2.3.2 Culturing conditions

Populations were grown in batch culture and were not axenic. Culture medium was prepared using 0.22 μm filtered Instant Ocean artificial seawater (salt concentration 30 ppt) aerated with 400 ppm CO_2 and supplemented with Keller and f/2 vitamins. Control cultures were maintained in a 14:10 hour light:dark cycle at 85 μmol photon m^{-2} s^{-1} and at a constant temperature of 18°C (Table 2.1).

Table 2.1. A comparison of the control environment and the environment treatments that were used for each environmental condition in this study.

Environment	Control	Treatment
Phosphate (μM)	10	5
Salt (ppt)	30	25
Light (μol m^{-2} s^{-1})	85	60
Temperature (°C)	18	20

For the selection experiment, *O. tauri* populations were grown without exposure to viruses in the control environment and four selection environments (Figure 2.2). The selection regimes used were higher temperature, lower light, lower phosphate and lower salinity (Table 2.1). Cultures were acclimated in each selection environment for one week, followed by one week of growth in each environment.
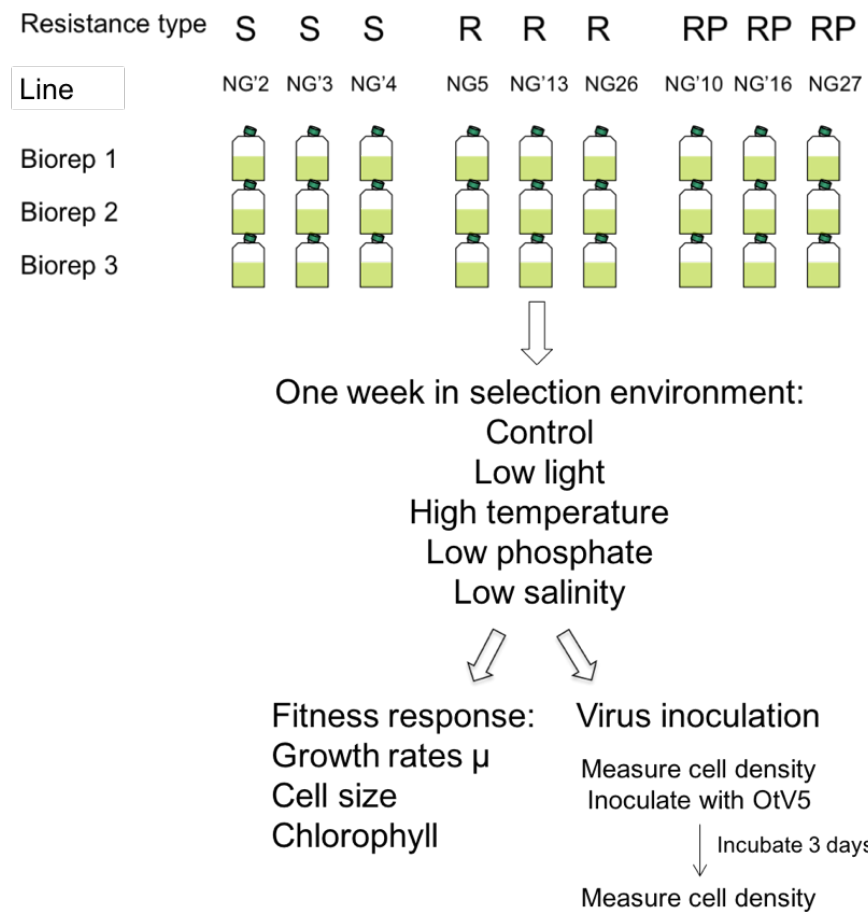
Figure 2.2. Experimental set-up. Three biological replicates each of three susceptible (S), three resistant (R) and three resistant producer (RP) *O. tauri* lines were grown under different environments for one week. Following one week in each selection environment, fitness and susceptibility to OtV5 were measured.

For the low phosphate environment, phosphate was reduced by preparing Keller media with only half the amount of β-glycerophosphate that would normally be used. Although the phosphate concentration in the low phosphate environment is not low compared to natural seawater [0.01– 2.99 μmol l^{-1} in the Leucate lagoon where *O. tauri* and OtV5 inhabit (Clerissi, Grimsley, Subirana, *et al.*, 2014)], it is low compared to the control media in which the populations had been maintained prior to the experiment. For culture medium with a lower salinity than the control, Instant Ocean was added to reach a salt concentration of 25 ppt. For the low light condition, culture flasks were wrapped in 0.15 neutral density foil to give a light intensity of

60 μmol m^{-2} s^{-1}. Cultures in the high temperature condition were maintained on a heat mat (Exo Terra Heat Wave substrate heat mat) set at 20°C.

2.3.3 The effect of viral exposure on cell division rates

Following one week of growth in the selection environment, each population was inoculated with a fresh suspension of OtV5 particles to test whether it was susceptible or resistant to viral lysis. Samples were tested by inoculating 1 ml cell culture at a density of 10^5 with 10 μl OtV5 in 48-well plates with three replicates for each sample. Controls that were not inoculated with viruses were used as a control for cell growth. Cell density was measured using a FACSCanto flow cytometer 3 days after inoculation.

2.3.4 Population growth of susceptible and resistant populations across different environments

Following the acclimation period, average cell densities per day of all cultures were measured over one week of growth in each environment. Cells were counted using a BD FACS-Canto II (BD Biosciences) flow cytometer before the first transfer and after seven days of growth. Each population was counted in triplicate. The cell counts were converted to cells per millilitre and the number of divisions per day was calculated using Eq. (2.1).

$$(2.1) \quad \mu(d^{-1}) = \frac{log_2(\frac{N_t}{N_0})}{t - t_0}$$

where N_t and N_0 are the cell densities (cells ml^{-1}) at times t_1 and t_0 (days). This measures the average number of cell divisions per ancestor over a single growth cycle and allows a comparison of offspring production between environments (Brennan and Collins, 2015). This is useful if different environments produce different growth curves since populations with different growth strategies can be compared. This calculation is also not sensitive to small differences in N_0, which is

important if the population size reached during the acclimation period differs between environments or resistance types.

2.3.5 Cell size and chlorophyll content of populations with different resistance types across environments

Cell size and relative chlorophyll content per cell volume were determined using a FACSCanto flow cytometer. Cell size was inferred from FSC (forward scatter), which was calibrated using beads of known sizes (1, 3 and 6.6 μm). Chlorophyll fluorescence was inferred by measuring PerCP-Cy5.5 emission with excitation at 488 nm. Relative chlorophyll was analysed by taking the average chlorophyll fluorescence for all susceptible populations in the control environment and setting this to a value of 1, with chlorophyll measurements of all populations relative to this value.

2.3.6 Statistical analysis

Data were analysed with linear mixed effects models using the statistical package nlme in R (version 3.2.0) to identify differences in growth rates between the different environments after one week of growth and after virus inoculation. Environment and resistance type were fixed effects when analyzing growth under different environments, and environment, resistance type and treatment were fixed effects when analysing virus inoculation under different environments. Line was a random effect in both models.

Post hoc mixed effects models were used to examine whether growth rate had an effect on cell size and chlorophyll content in cells. Environment, resistance type and growth rate (cells divisions per day) were set as fixed effects with line as the only random effect.

2.4 Results

2.4.1 The effect of viral exposure on cell division rates depends on resistance type

After one week of growth in a novel environment, all *O. tauri* populations were inoculated with OtV5 and cell densities were measured three days later. All R and RP lines remained resistant to lysis and S lines remained susceptible (Figure 2.3). A significant interaction between environment, resistance type and treatment (OtV5 inoculation) was observed to affect susceptibility to virus inoculation (ANOVA environment $\times$ resistance type $\times$ virus treatment, $F_{8,234}$ = 2.91, p = 0.0041). Susceptibility of *O. tauri* to OtV5 was driven by resistance type, as expected (ANOVA, resistance type $\times$ virus treatment, $F_{2,234}$ = 360.14, p < 0.0001). OtV5 inoculation had a significant effect on cell density ($F_{1,234}$ = 361.62, p < 0.0001), since populations of S cells fell to almost zero. No difference was observed in resistance between R and RP populations (t = 0.46, p = 0.66).
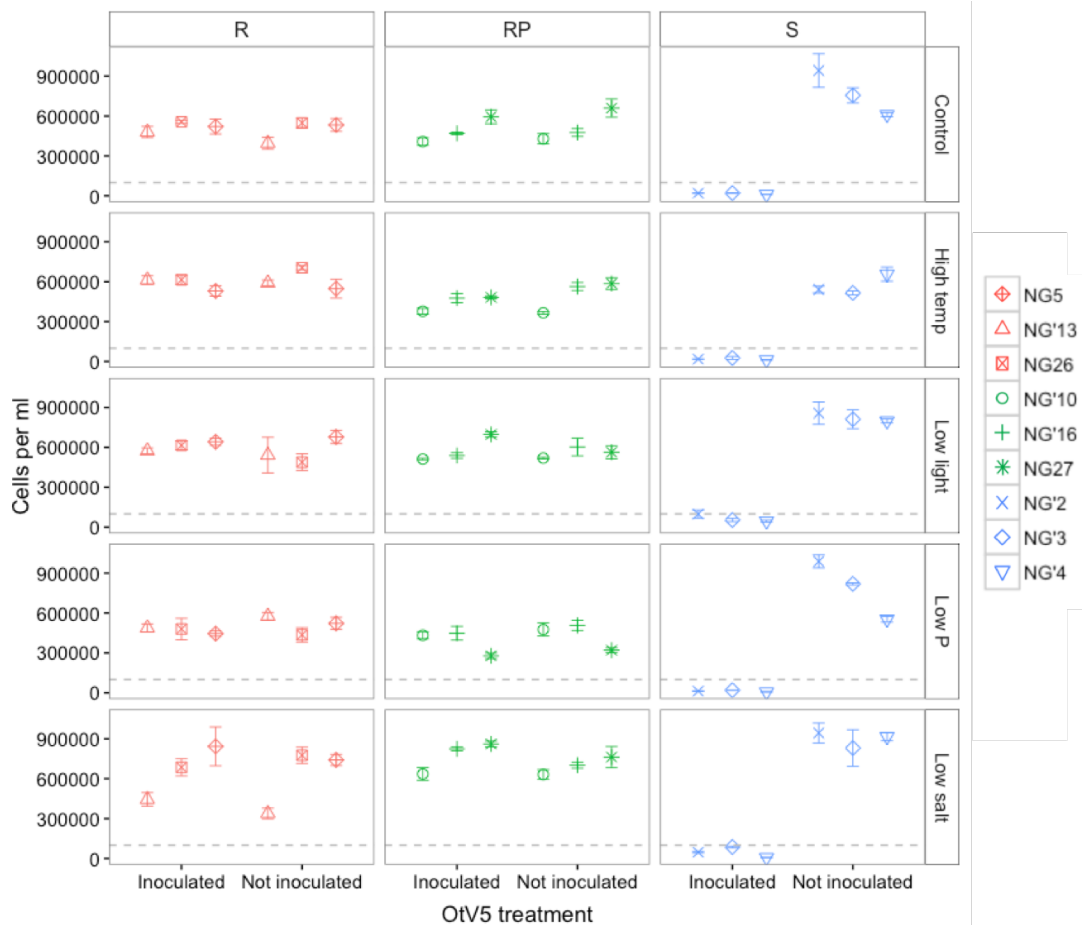
Figure 2.3. Mean (± SEM) cell density ml^{-1} of resistant (R), resistant producer (RP) and susceptible (S) *O. tauri* lines three days after OtV5 inoculation in five environments. Points represent the average of the three biological replicates for each line. Inoculated = cells inoculated with OtV5, Not inoculated = negative control cultures that were grown for the same period of time, but not inoculated with OtV5. The dashed line represents the starting densities of the cultures at 10^5 cells ml^{-1}.

The effect of virus inoculation did not vary with environment (ANOVA, environment × virus treatment, $F_{4,234} = 0.89$, $p < 0.46$). However, environment alone had a significant effect on cell density ($F_{4,234} = 26.01$, $p < 0.0001$), because of some populations having particularly high growth rates in the control and low salt environments. For both R and RP cells, there were cases where virus inoculation resulted in higher growth rates than the non-inoculated controls (Figure 2.3). Cell densities were repeatedly higher in one inoculated line (NG'13) than the control in the low salt environment in R cells and in the low light environment for one line

(NG27) in RP cells. This indicates that cell growth can increase in response to viruses in resistant populations. This occurs consistently in all replicates of a given line when it happens, but does not occur in all lines of a resistance type. I also see cases where lysis in some populations of S cells is incomplete, notably in the low light (NG'2) and low salt (NG'3) environments. Again, this does not occur in all lines, but it occurs reliably in replicates of the same line. While these effects of environment on lysis are not statistically significant because they do not occur over all populations within a resistance type, it could have evolutionary and ecological effects on the occasions when it does occur, which I discuss below.

2.4.2 Growth rate varied across environments regardless of resistance type

All populations were grown in a novel environment in the absence of OtV5 for one week, over which growth rate was measured. The response of *O. tauri* growth to the environment depended on resistance type (effect of environment × resistance type, $F_{8,114} = 4.45$, $p = 0.0001$). Growth rates were higher in the control environment except for a single RP line, NG'10, which divided rapidly in the low salt environment (Figure 2.4). Populations grown in the low phosphate environment all had reduced growth rates and showed less variation in growth than in all other environments.
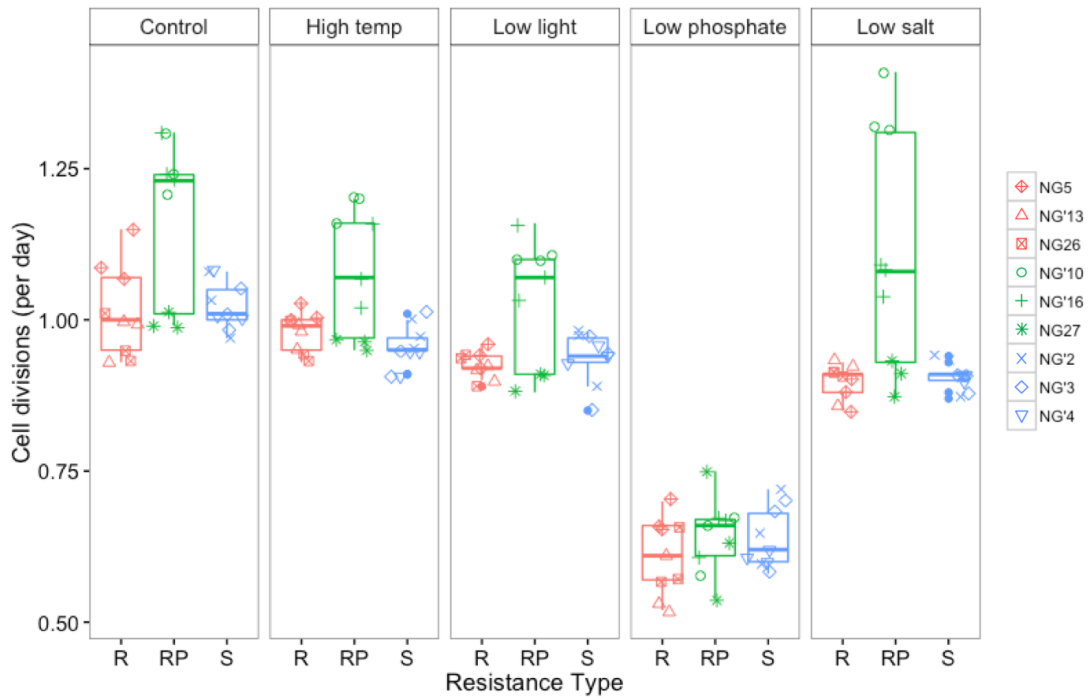
Figure 2.4. Mean growth rates, measured as average number of cell divisions per day over 7 days, of susceptible (S), resistant (R) and resistant producer (RP) *O. tauri* cells grown in five environments in the absence of OtV5. There were three lines for each resistance type, with three biological replicates for each line. Boxes represent the interquartile range with the median indicated as the thick line inside the box, and whiskers extend to the highest and lowest values within 1.5 × the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

2.4.3 The effect of resistance type on growth depends on environment

Resistance type alone did not significantly affect the growth rate of *O. tauri* ($F_{2,6} = 2.88$, $p = 0.1328$). This is because S and R lines and one RP line had similar population growth rates in all environments (Figure 2.4). In contrast, two RP lines had higher growth rates than both R and S cells, with populations consistently showing elevated growth rates. Two out of the three RP lines, NG'10 (shown by circle in Figure 2.4) and NG'16 (shown by cross in Figure 2.4), had higher growth rates than S and R cells in four out of the five environments ($F_{3,5} = 17.19$, $p = 0.046$).

The single exception was the low phosphate environment, where all resistance types had similar low growth rates. These data indicate that there is either no cost or an undetectable cost of resistance in terms of growth to either infection or lysis over a range of environments and that there can be a growth benefit of being resistant to lysis in some environments, as evidenced by the rapid growth of some RP populations.

2.4.4 Populations resistant to lysis can have a growth advantage in some environments

In order to assess whether the S, R and RP resistance types responded similarly to the different environments, environments were ranked from best to worst, based on population growth rates. All resistance types displayed highest growth rates in the control environment and the lowest growth rates in low phosphate (Table 2.2). R lines had the same rank order of environments as the S lines. Since the growth rates of the RP lines were highly variable relative to the other resistance types, containing two lines that grew quickly, the RP lines were grouped into "fast-growing" (NG'10 and NG'16) and "normal-growing" (NG27). RP lines showed the same rank order of environments for both the fast and normal growing populations, except in low salt for the fast-growing populations. This was due to one population (NG'10) displaying exceptionally high growth (Figure 2.4). Growth rate was the same in the low salt and low light environments for the normal-growing RP population. Fast-growing RP cells had higher cell growth in all environments except low phosphate.

Table 2.2. Ranked environments by fitness as measured by cell divisions per day for each resistance type. Environments were ranked in order from best to worst, where 1 is the environment with the highest growth rate. Fast and normal growing resistant producers were ranked separately to compare slopes.

Resistance Type	Rank	Environment
S	1	Control
S	2	High temperature
S	3	Low light
S	4	Low salt
S	5	Low phosphate
R	1	Control
R	2	High temperature
R	3	Low light
R	4	Low salt
R	5	Low phosphate
RP	1	Control
RP	2	High temperature
RP	3	Low salt
RP	4	Low light
RP	5	Low phosphate
RP fast	1	Control
RP fast	2	Low salt
RP fast	3	High temperature
RP fast	4	Low light
RP fast	5	Low phosphate

To measure how sensitive growth rates were to environmental change, the slopes of the ranked environments were compared (Figure 2.5). The two fast-growing RP lines had a higher intercept (ANOVA effect of rank on growth, $F_{1,125} = 1112.56$, $p < 0.0001$), demonstrating the increase in growth rate compared to the other populations.

These data show that faster growing populations had a growth advantage in environments that allowed reasonable growth for all resistance types, however in the lowest ranking environment (which was low phosphate for all resistance types), these populations grew equally badly.
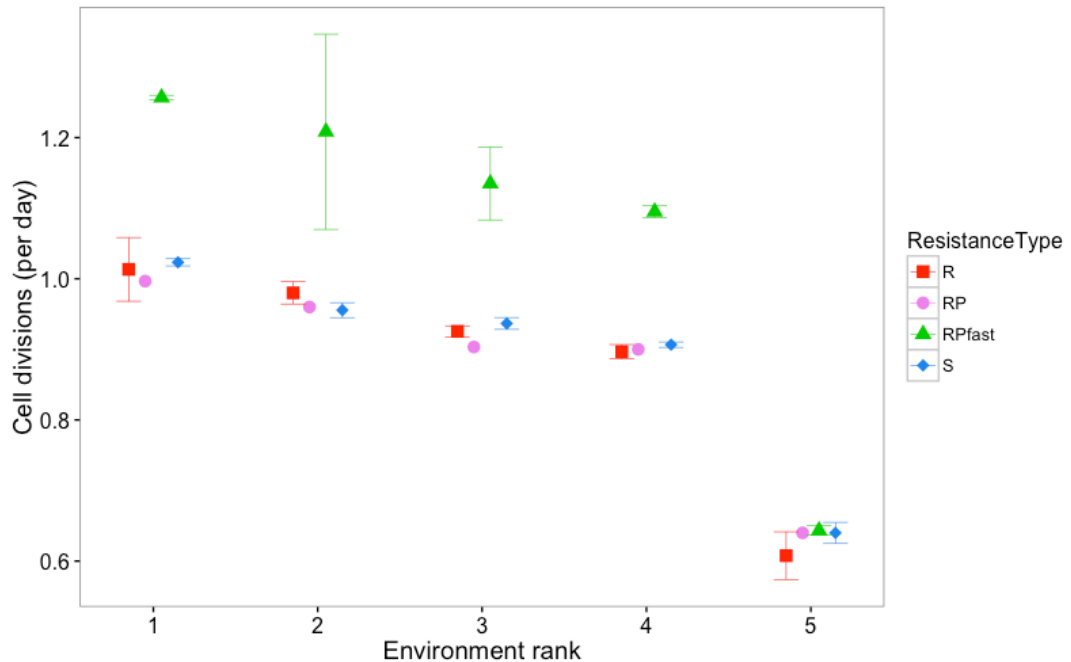
Figure 2.5. Ranked environment by average cell divisions per day over 7 days (± SEM) for susceptible (S), resistant (R) and resistant producer (RP and RPfast) cells. Environments were ranked in order from best to worst for each resistance type based on growth rate in the absence of OtV5, where 1 is the environment with the highest growth rate. Fast and normal growing resistant producers have been plotted separately for visual purposes.

2.4.5 Size and chlorophyll content vary between cells with different resistance types in response to environment

After one week of growth in a novel environment without viruses, cell size and relative cell chlorophyll content were measured. Response of resistance type on cell size depended on environment (effect of environment × resistance type, $F_{8,114} = 5.48$, $p < 0.0001$). Cells were larger under low light ($t = 3.83$, $p = 0.0002$) and low

phosphate conditions ($t = 7.49$, $p < 0.0001$), compared to the control environment (Figure 2.6). No significant effect of resistance type alone was observed on cell size ($F_{2,6} = 0.01$, $p = 0.9945$). However, under low phosphate, there was a large variation in cell size between the fast and normal growing RP populations.
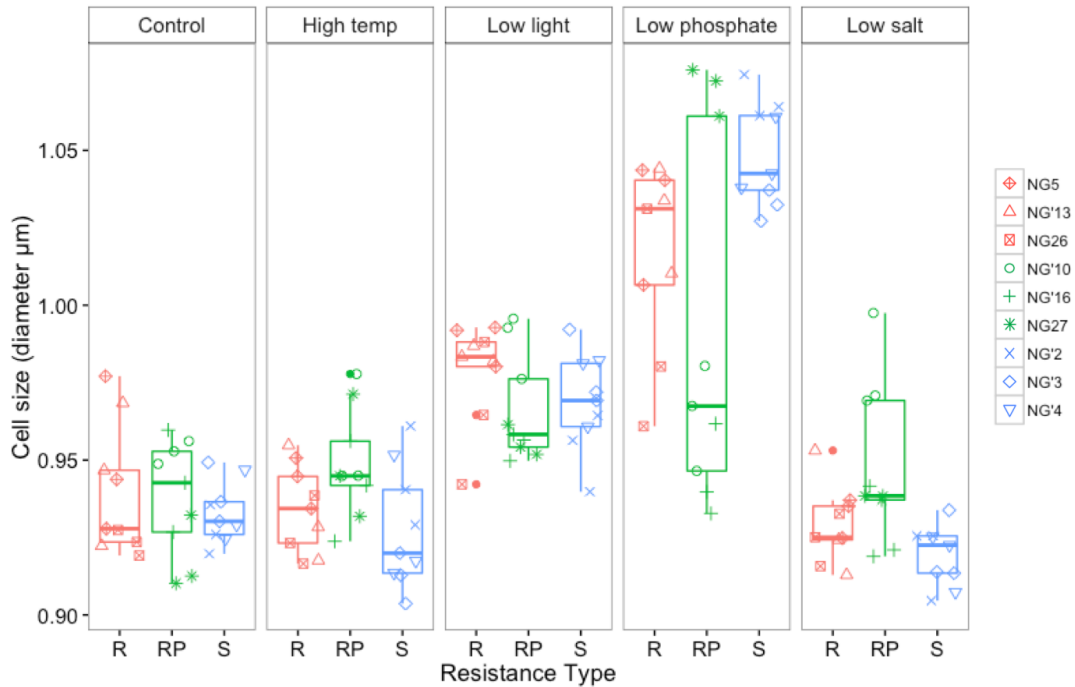
Figure 2.6. Mean cell size for susceptible (S), resistant (R) and resistant producer (RP) cells after seven days of growth in the absence of viruses in five environments. There were three lines for each resistance type, with three biological replicates for each line. Boxes represent the interquartile range with the median indicated as the thick line inside the box, and whiskers extend to the highest and lowest values within $1.5 \times$ the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

The two fast-growing RP lines had smaller cells than the normal growing RP line in the low phosphate environment. The RP line with normal growth had cells that were similar in size to the S lines (Figure 2.6). To examine whether fast growing RP lines had different cell sizes than did lines with normal growth rates, post hoc models were used to analyse the two fast-growing lines separately. Overall, no significant effect of resistance type was observed on cell size when normal and fast growing RP populations were analysed separately (ANOVA $F_{2,6} = 0.22$, $p = 0.8812$).

Additionally, a model examining growth rate as a fixed effect was performed. This showed a significant interaction between resistance type and growth rate ($F_{2,99}$ = 4.64, p = 0.01). However, the statistical power in this data set, which contained only one line of normal growing RP cells and two lines of fast growing RP cells, was low, such that the chances of detecting an effect of resistance type on cell size is unlikely here even if one exists (power = 0.142).

The effect of resistance type on chlorophyll content per cell volume depended on environment (effect of environment × resistance type, $F_{8,114}$ = 10.68, p < 0.0001). Under low light, chlorophyll varied little between the three resistance types (Figure 2.7). In the other environments, S and R strategies usually displayed similar chlorophyll content levels with RP displaying lower chlorophyll levels in all environments except low phosphate.

By inspection, I see that the fast-growing RP lines have less chlorophyll per cell volume than the normal growing RP line in all environments except low phosphate (Figure 2.7). I used a post hoc model with growth rate as a fixed effect to investigate whether the fast-growing RP populations also had different chlorophyll contents. Growth rate had an effect on chlorophyll content, with fast growing RP populations having lower chlorophyll content, and the effect of growth rate was dependent on environment ($F_{4,99}$ = 3.85, p = 0.01) and resistance type ($F_{2,99}$ = 6.27, p = 0.003). This suggests that the growth rate of the fast-growing RP populations reduced chlorophyll content.
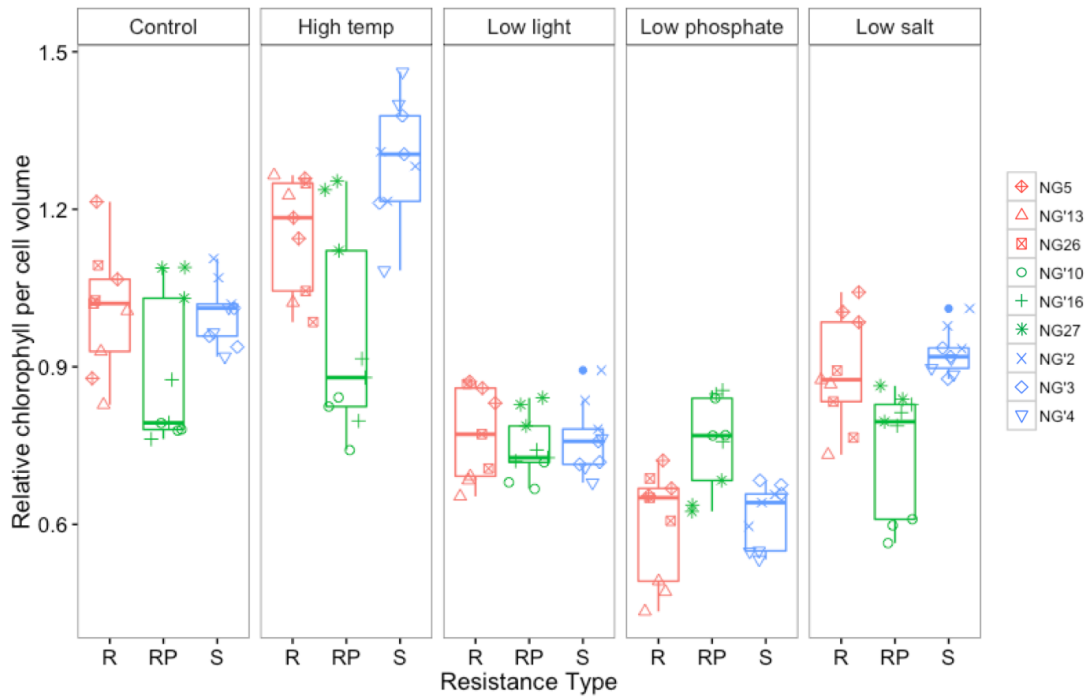
Figure 2.7. Mean relative chlorophyll to cell size for susceptible (S), resistant (R) and resistant producer (RP) cells after seven days of growth in the absence of viruses in five environments. There were three lines for each resistance type, with three biological replicates for each line. Boxes represent the interquartile range with the median indicated as the thick black line inside the box, and whiskers extend to the highest and lowest values within 1.5 × the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

2.5 Discussion

2.5.1 Effect of environment on host resistance

I observed no differences in susceptibility of any of the lines to OtV5 over the environments tested, i.e. S cells remained susceptible to lysis and R and RP cells remained resistant to lysis. While the ability of the virus to lyse host cells did not depend on the environment, R and RP cells had different growth responses to viral exposure. There were two cases in which a resistant population repeatedly had a higher cell density after exposure to OtV5 than its paired control culture that was not inoculated. I speculate that this may be a response to the virus, which causes the host

cells to divide more rapidly. This would be advantageous if, for example, a population that was made up of mixed susceptible and resistant cells were exposed to viruses – any resistant cell lineages that could increase their growth rate would then take over the population by overgrowing any remaining resistant cells whose growth rate was unaffected by exposure to virus.

I did not detect a growth cost of resistance when R and RP populations were grown in the absence or presence of OtV5 after exposure to a novel environment. A trade-off for being resistant to viral infection is expected, because if there were no cost there should be a strong selection pressure for all cells to become resistant, yet we still find susceptible populations both in the laboratory and in the ocean (Thomas *et al.*, 2011; Clerissi *et al.*, 2012). Previous work shows that susceptible cells can have a competitive advantage against resistant cells (Lenski, 1988a). Additionally, I speculate that resistance to one virus strain could make these cells susceptible to other OtVs. Clerissi *et al.* (2012) showed that OtVs are mainly intraspecies-specific and that hosts that are the most resistant to infection can often be infected by more generalist viruses. This specificity could be caused by proteins involved in adaptive behaviour (Clerissi *et al.*, 2012). Thus, I suggest that in addition to the abiotic environment, biotic environment could play a large role in *O. tauri* resistance strategy.

Since viruses are responsible for a large proportion of microbial death, there is strong selection on hosts for resistance or tolerance to viral infection. There are several suggestions to explain the paradox of how susceptible algal cells and their viruses are able to co-exist in marine environments without extinction of the host. One theory is that there must be a cost to being resistant to infection. This is often expected to be a reduction in growth (Weinbauer, 2004), and has been observed in *Synechococcus*, in which there was a 20% reduction in fitness compared to the ancestor in resistant strains (Lennon *et al.*, 2007). Thus, in the absence of viruses, resistant cells can have a lower fitness. This could lead to decreased numbers in the absence of viruses. An evolutionary 'arms race' may occur when viruses and their hosts adapt reciprocally to overcome resistance and infection, respectively. I find little evidence for a cost of

resistance in my study, but this may be because the laboratory environments used are missing a key aspect of the natural environment that, if present, results in a cost of resistance in *O. tauri*. Alternatively, although deviating from the standard control environment, none of the environments in this study were severely stressful, with even the low phosphate environment allowing reasonable growth. Thus, it is possible that I did not detect a growth cost because the changes to the environments used were relatively modest.

Various strategies for virus resistance have been reported in phytoplankton, from internal to external mechanisms (Tarutani *et al.*, 2006; Bidle *et al.*, 2007; Brussaard *et al.*, 2007; Stoddard *et al.*, 2007; Frada *et al.*, 2008). *O. tauri* cells acquire resistance through chromosomal restructuring (Yau *et al.*, 2016), the first reported observation of a resistance mechanism of this kind. I found that short-term exposure to novel environments does not affect resistance type and I did not observe any cost of resistance leading to cells losing their resistance to OtV5.

2.5.2 Effect of resistance type and environment on population growth and other phenotypic traits

I found that after one week in a novel environment, growth rate of *O. tauri*, as measured by the average number of cell divisions per day over seven days, varied across environments for all resistance types. RP populations had the fastest average cell division rates in most environments. Resistance types mainly showed the same environmental preferences, with average cell division rates highest in the control environment. The only exception was one RP population that divided rapidly in the low salt environment. The lowest growth rates were observed in the low phosphate environment, which was expected since these cells were deprived of a key nutrient.

Two of the three RP lines divided more rapidly than all of the S and R lines. These lines were fast-growing in many environments, including the control environment, suggesting that the rapid growth is a general character of these two RP populations, rather than a response to stress or novelty. This faster growth rate in RP lines relative

to S and R lines was not observed in previous studies on *O. tauri* by Thomas *et al.* (2011) who detected no difference in growth rate between S, R and RP cells. Thus, I did not observe a fitness cost in terms of growth rate for the remaining populations since S, R and normal-growing RP populations had similar growth rates across environments. This was expected, at least in the control environment, where previous studies have only been able to detect a minimal cost of resistance by using direct competitions (Thomas *et al.*, 2011). Interestingly, the two fast-growing RP populations could not be detected as having more rapid growth under low phosphate, however these populations responded differently in their size and chlorophyll contents.

Reduced growth rate has been observed as a cost of resistance in several microbial species (Lennon *et al.*, 2007; Haaber and Middelboe, 2009). However, in my experiment there was no environment in which resistant cell types grew at slower rates than S cells. In fact, I observed the opposite in two out of the three RP lines, where resistant cells grew faster than the S populations across all environments except low phosphate. In cases where resistant cells (R or RP) did not divide faster than susceptible ones, they divided at the same rate. Taken together, this suggests that the cost of resistance to OtVs is likely to be small or absent. This opens the question of how the acquisition of resistance to OtVs affects both host and viral ecology.

Environment affected cell size, whereas generally, resistance type did not. However, under low phosphate, the two fast growing RP lines were smaller than the normal growing RP line, suggesting that under nutrient limitation these cells were able to divide at a smaller cell size. Smaller phytoplankton cell size is often selected for in nutrient limited environments since smaller cells have a larger surface area to volume ratio and a thinner diffusion boundary layer, thus facilitating nutrient uptake (Finkel *et al.*, 2010; Peter and Sommer, 2015). Although fast-growing RP lines in this selection environment were smaller than the normal growing RP line, their cell size was not different from the fast-growing RP lines in the other environments. The control was the only environment in which fast growing RP populations were larger

than the normal growing populations, indicating that there may be a (direct or indirect) fitness benefit associated with the increased size of the RP type under control conditions.

In contrast to previous studies, all populations in the low phosphate environment, except fast growing RP, increased in cell size. Cell division of larger phytoplankton cells requires greater nutrient concentrations, which can decrease the division rate. Since cells in the low phosphate environment had a reduced growth rate in terms of cell divisions, this could have resulted in cells that reached a larger volume even though the environment was phosphate-poor. It has previously been suggested that increasing algal cell size, and thus the volume to surface area ratio, can facilitate reduced phosphorus uptake under phosphate-limited conditions, and that this adaptation response may be more favourable than decreasing cell size (Šupraha *et al.*, 2015). A common response of coccolithophores to phosphate limitation is reduced growth rate and increased cell size (Šupraha *et al.*, 2015).

Smaller phytoplankton cells have often been observed growing at higher temperatures in natural environments, which is thought to arise from the temperature-size rule (e.g. Atkinson *et al*. 2003, Morán *et al*. 2010). These studies used large temperature ranges, but there was no effect of the modest increase in temperature on cell size in this study. Smaller cells have also been reported to cope better with both light limitation and light saturation compared to larger cells due to a reduction in internal shading (Geider *et al.*, 1986; Raven, 1998; Finkel *et al.*, 2010). I found no significant difference in cell size under low light, although there was a non-significant trend for cells to be slightly larger in low than under control light.

Environment was found to have a significant effect on chlorophyll content per cell volume, whereas resistance type alone had no effect. I observed lower chlorophyll per cell volume in all environments compared to the control except high temperature. Although resistance type alone did not have an effect, growth rate had a significant effect on chlorophyll content per cell volume when included in the model and normal growing S, R and RP cells in the control and high temperature environments had the

highest chlorophyll levels across all environments. In contrast, fast-growing RP lines showed no significant difference in chlorophyll content per cell volume across all five environments. All lines had their lowest growth rates in the low phosphate environment and cells in this environment had the lowest chlorophyll content, except for fast growing RP lines. Fast growing RP lines had lower chlorophyll content than the normal growing RP population in all environments except for low phosphate.

One experiment using cultures of different phytoplankton groups found that chlorophyll content was lower during both nitrogen and phosphorus depletion (Riemann *et al.*, 1989). Additionally, phytoplankton cells grown under low nutrients have been observed to decrease their photosynthesis rates (Litchman *et al.*, 2003; Spilling *et al.*, 2015). This may be due to the cells allocating resources to synthesizing chloroplasts under nutrient limitation. In my study the control environment was the preferred one, and it is possible that cells were unable to synthesise large quantities of chlorophyll in the other (less permissive) environments since their energy was allocated to growth. It is possible that under elevated temperature, the metabolism of *O. tauri* was increased, leading the cells to synthesise more chlorophyll. Temperature did not affect chlorophyll *a* content in diatoms (Sigaud and Aidar, 1993). Salinity appears to affect different phytoplankton species differently, with some species showing no change in chlorophyll content across a range of salinities, and others having higher chlorophyll contents at the optimum salinity for growth (McLachlan, 1961; Sigaud and Aidar, 1993).

2.6 Concluding remarks

Resistance of microbes to virus infection often comes at a cost, with one common observation being a reduction in growth compared to susceptible cells in the population. In this study, my aim was to measure resistance to viruses in *O. tauri* across different environments and to determine whether strength of resistance depends on environmental context. I did not observe a cost of resistance as measured by cell division rate, cell size or chlorophyll content in the present study. Growth rates of *O. tauri* were reduced when grown in low phosphate, however this did not

affect the ability of OtV5 to lyse susceptible cells in this environment. Additionally, although growth rates were lower than the controls in high temperature, low light and low salinity, OtV5 still caused cell lysis of susceptible cells. Indeed, some RP populations had evolved high growth rates, and some also increased their growth rates after exposure to viruses. Both observations suggest that resistance strategy could have interesting ecological consequences by changing the relative fitness of different populations.

3. Virus resistance is not costly in *Ostreococcus tauri* evolving under different environmental stressors

3.1 Abstract

Viruses are important evolutionary drivers of host ecology and evolution. The marine picoplankton *Ostreococcus tauri* has three known resistance types that arise in response to infection with the Phycodnavirus OtV5: susceptible cells (S) that lyse following viral entry and replication; resistant cells (R) that are refractory to viral lysis; and resistant producers (RP) that are mainly resistant but maintain some viruses within the population. To test for evolutionary costs of maintaining antiviral resistance, I examined whether *O. tauri* populations composed of each resistance type differed in their evolutionary responses to several environmental drivers (lower light, lower salt, lower phosphate, higher temperature, and a changing environment) in the absence of viruses for approximately 200 generations. I did not detect a cost of resistance as measured by life-history traits (population growth rate, cell size and cell chlorophyll content) and competitive ability. Specifically, all R and RP populations remained resistant to OtV5 lysis for the entire 200-generation experiment, whereas lysis occurred in all S populations, suggesting either that resistance is not costly to maintain even when direct selection for resistance was removed, or that there could be a genetic constraint preventing return to a susceptible resistance type, or both. Following evolution, all S population densities dropped when inoculated with OtV5, but not to zero, indicating that lysis was incomplete, and that some cells may have gained a resistance mutation over the evolution experiment. These findings suggest that maintaining resistance in the absence of viruses was not costly.

3.2 Introduction

Viruses are the most abundant biological entities in the oceans, with an estimated 10^{30} particles globally (Suttle, 2007). Viruses play a key role in marine food webs, partially because viral infection of unicellular organisms often results in cell lysis, where the infected cell bursts to release the new viruses; products of lysis feed back into the microbial loop and provide organic matter to organisms at the base of the food web daily (Wilhelm and Suttle, 1999). In addition to being a large cause of mortality to their hosts, viruses can exert strong selection on host immune defence, leading to the evolution of host resistance mechanisms. Strong immune defences, in turn, impose strong selection on viruses to evade these resistance responses leading to an ongoing co-evolutionary process between hosts and viruses (Koskella and Brockhurst, 2014). Experimental evidence of host-virus coevolution has come mainly from bacteria-phage systems (Dennehy, 2012; Koskella and Brockhurst, 2014). Viruses evolve rapidly due to their high mutation rates (Flint *et al.*, 2000) which can strongly influence the evolution of their hosts. However, in addition to infection, hosts are also subject to other selection pressures, such as environmental changes. In the case of marine hosts, they will be subject to natural selection both from their viruses, and from, for example, the changes in nutrients, temperature and light associated with global change in the oceans (Doney *et al.*, 2012), which opens up the possibility that the genetic and physiological changes associated with resistance may affect host evolution in response to challenges other than the virus itself. This in turn has the potential to affect how primary productivity at the base of the marine food web evolves in response to global change.

Studies have examined environmental effects on interactions between microalgae and their viruses under a range of conditions including changes in temperature (Nagasaki and Yamaguchi, 1998; Wells and Deming, 2006), nutrients (Bratbak *et al.*, 1993, 1998; Wilson *et al.*, 1996; Bellec, Grimsley, Derelle, *et al.*, 2010; Maat *et al.*, 2014), UV radiation (Jacquet and Bratbak, 2003), light intensity (Bratbak *et al.*, 1998; Jacquet *et al.*, 2002; Thyrhaug *et al.*, 2002), and CO_2 levels (Larsen, A. Larsen, *et al.*, 2007; Chen *et al.*, 2014; Maat *et al.*, 2014). Environmental change can

have direct effects on marine viruses, for example by damaging and/or deactivating the particles through UV exposure or extreme temperatures (Jacquet and Bratbak, 2003; Wells and Deming, 2006). However, viral abundance is thought to be mainly dependent on host availability and, therefore, the effects of environmental change on viruses are expected to be mainly indirect (e.g. Danovaro *et al*. 2011). In this chapter I focus on host evolution rather than viral selection.

Hosts are capable of evolving resistance to their viruses, though resistance often entails a fitness cost, which can vary in form and magnitude (Bohannan *et al.*, 2002). Costs of resistance that have been reported in microorganisms include reduced competitive ability (Lenski, 1988a; Bohannan *et al.*, 2002), reduced growth rate (Lennon *et al.*, 2007; Frickel *et al.*, 2016), reduced original function of a receptor protein (Seed *et al.*, 2012; León and Bastías, 2015), and increased susceptibility to other viruses (Avrani *et al.*, 2011; Clerissi *et al.*, 2012; Marston *et al.*, 2012). If the cost of resistance is substantial and related to growth or competitive ability, resistance might be lost when the selection pressure for it is removed (i.e. when viruses are absent) (Meyer *et al.*, 2010). This is because when viruses are present and able to interact with their host cells, resistant hosts should have a selective advantage over susceptible hosts by avoiding lysis. However, in the absence of viruses, the selection pressure for resistance is removed and costs of resistance, if present, should reduce host fitness, so that there is an advantage to losing resistance. Most studies have focused on costs of resistance in bacteria (e.g. Lennon *et al*. 2007, Avrani *et al*. 2011, 2012, Avrani & Lindell 2015), and data for eukaryotic microalgae are lacking, which limits our ability to translate the literature on host-virus interactions to primary producers in the oceans. Because marine phytoplankton are the dominant primary producers in oceans (Field, 1998), changes in the abundance, distribution and composition of microalgal assemblages in response to climate change are likely to have important implications for marine communities.

The marine picoeukaryote *Ostreococcus tauri* and its viruses, *Ostreococcus tauri* viruses (OtVs), are abundant in Mediterranean lagoons (Bellec *et al.*, 2009). OtVs are lytic viruses belonging to the family *Phycodnaviridae* that cause susceptible (S)

host *O. tauri* cells to burst following infection (Derelle *et al.*, 2008). However, two resistant host types have been identified (Thomas *et al.*, 2011; Yau *et al.*, 2016). In the first type, viruses can attach to the resistant (R) host cells but are unable to replicate and cause lysis. In the second type, resistant producer (RP) populations consist mainly of resistant cells with a minority of susceptible cells (<0.5%) that maintains a population of viruses. These two resistance mechanisms have been observed repeatedly and remain resistant to lysis over many generations of sub-culturing (Thomas *et al.*, 2011; Yau *et al.*, 2016). Previous work found that there was no difference in growth rates between the three resistance types when they were maintained separately under standard laboratory culturing conditions, although long term competitions indicated a cost of resistance with susceptible cells outcompeting resistant cells and resistant cells outcompeting resistant producers after 100 and 200 days, respectively (Thomas *et al.*, 2011). In Chapter 2, I showed that being resistant to lysis can sometimes even incur a growth advantage (Heath and Collins, 2016).

In this study, I examined whether a cost of resistance could be detected in *O. tauri* in terms of the ability to adapt to different environmental conditions, and whether the evolutionary responses to environmental change were affected by resistance type. Populations of S, R and RP *O. tauri* were evolved under different environmental conditions in the absence of viruses for 200 generations to examine whether resistance type was maintained and how resistance type affected evolutionary responses, even in the absence of coevolutionary dynamics imposed by the presence of viruses. I found that all R and RP populations remained resistant to OtV5 inoculation across all environments, whereas S populations had a lower proportion of cell lysis at the end than at the start of the evolution experiment. Additionally, resistance type affected cell division rates, size and chlorophyll content, whereas selection environment affected cell division rates and competitive ability.

3.3 Materials and Methods

3.3.1. Susceptible and Resistant Lines

O. tauri lines were obtained from N. Grimsley, Observatoire Océanologique, Banyuls-sur-Mer, France. Three susceptible lines (NG'2, NG'3 and NG'4), three resistant lines (NG5, NG'13 and NG26) and three resistant producer lines (NG'10, NG'16 and NG27) were used. All lines were derived from a single clone of *O. tauri* (RCC4221) and therefore had the same starting genotype.

3.3.2 Culturing Conditions

For each of the nine lines listed above, three biological replicates were evolved per environment (27 independent populations in total per environment). I refer to each independent replicate as a population. Populations were grown in batch culture that did not contain antibiotics and therefore were not axenic. Culture medium was prepared using 0.22 μm filtered Instant Ocean artificial seawater (salt concentration 30 ppt) supplemented with Keller and f/2 vitamins (Keller *et al.*, 1987). Control cultures were maintained in a 14:10 hour light:dark cycle at 85 μmol photon m^{-2} s^{-1} at a constant temperature of 18°C (Table 1). Each population was grown in 20 mL media and each week, 200 μL was transferred to fresh media to ensure populations were always growing exponentially. Cultures were re-suspended by gentle shaking every 2–3 days to prevent cells sticking to the bottom of the flask. For the evolution experiment, *O. tauri* populations were grown either in the control environment as described above, in low light, low phosphate, low salt or high temperature (Table 3.1), or a changing (random) environment in which one of the environments from those listed was chosen at random at each transfer. I refer to the environments where the populations evolved as "selection environments". Populations were grown in the absence of viruses for 32 weeks, corresponding to approximately 200 generations. A diagram of the experimental set-up is outlined in Figure 3.1.

Table 3.1. A comparison of the control environment and the treatments used for each selection environment used in this study.

Environment	Control	Treatment
Phosphate (μM)	10	5
Salt (ppt)	30	25
Light (μmol m^{-2} s^{-1})	85	60
Temperature (°C)	18	20

For the low light environment, culture flasks were wrapped in 0.15 neutral density foil to reduce light intensity. For the low phosphate environment, phosphate was reduced by preparing Keller medium with half the amount of β-glycerophosphate present in the control media. For low salt, Instant Ocean was added to reach 25 ppt. Cultures in the high temperature environment were maintained on an Exo Terra Heat Wave substrate heat mat set at 20°C. These selection environments were chosen so that the populations responded to them by changing their growth rates relative to the control environment. In batch culture, rapid growth is favoured by natural selection, so any environment that decreases growth rates should then result in natural selection for traits that will allow cell division rates to recover in that environment. However, the selection environments were not extreme, so that populations were still able to grow at a measurable rate and survive the dilution rate of the experiment. This is in part so that a similar number of generations elapsed in all environments over the course of the experiment.
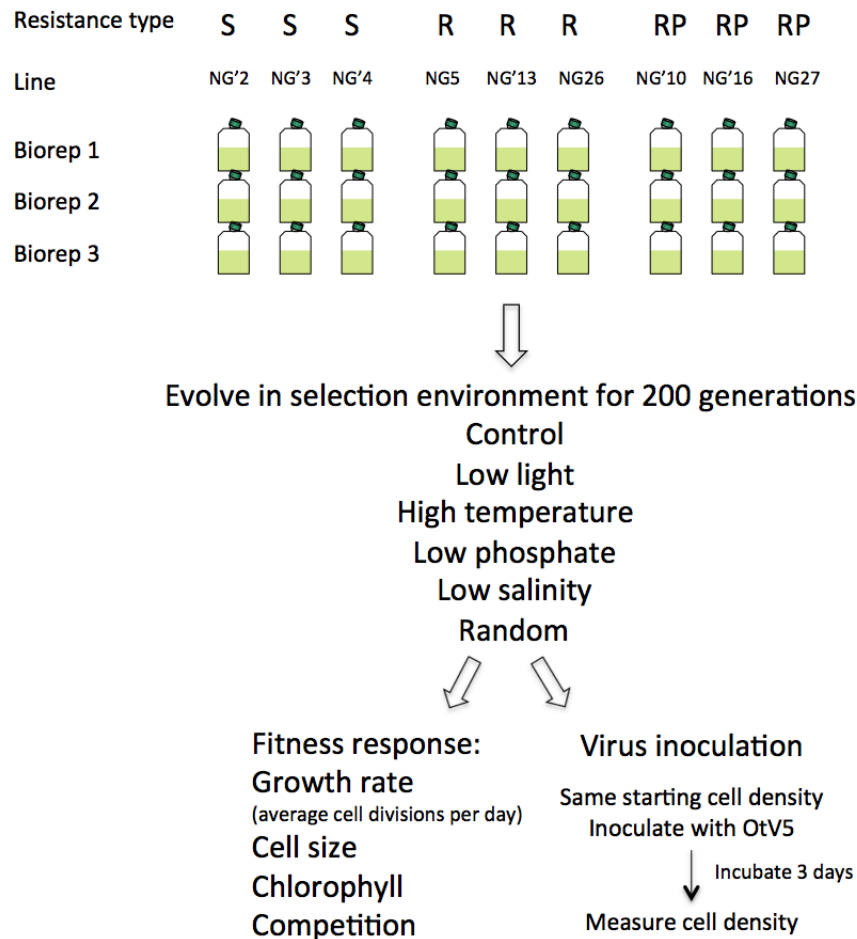
Figure 3.1. Experimental design of the selection experiment. Three lines of each *O. tauri* resistance type (S, R and RP) were evolved in different environments for approximately 200 generations. After evolution, different fitness traits were measured and populations were tested for susceptibility to OtV5 inoculation.

3.3.3 Testing RP Lines for Viral Production

All resistant producer (RP) lines were tested for viral production prior to the start of the experiment. To check whether the three RP lines (NG'10, NG'16 and NG27) were producing infectious viruses, I used the supernatant of each line to infect susceptible *O. tauri* strain RCC4221. Two millilitres of each population were transferred to an Eppendorf tube and centrifuged at 4000 × *g* for 15 min. Four hundred millilitres of the supernatant were removed carefully without drawing up

any of the cells from the pellet at the bottom of the tube, and used to inoculate 1 mL of susceptible *O. tauri*. OtV5 was used as a positive control and Keller media was used as a negative control. Eight replicates were performed before the experiment was started. The test was performed every four weeks with three replicates per population. Samples were checked for lysis either by observing by eye whether they were green or clear, or by measuring cell densities using a BD FACSCanto II (BD Biosciences) flow cytometer.

In addition to liquid lysis tests, frozen stocks of RP supernatant were made by adding dimethyl sulfoxide (DMSO) (final concentration 10%) and storing at −80°C. I tested these samples for viruses using the plaque assay technique (Derelle *et al.*, 2008). A 1.5% agarose suspension was made and 5 mL aliquots were prepared in Falcon tubes and held at 70°C in a water bath. In a 50 mL Falcon tube, 30 mL exponentially growing *O. tauri* culture, 15 mL Keller media and 5 mL agarose were mixed rapidly but gently by inverting the tube (final agarose concentration 0.15%). The agarose was poured into a 12 cm square petri dish and left to set. Tenfold serial dilutions of the RP supernatant were made in 96-well plates using one row per sample. A Boekel Replicator was used to transfer all of the serial dilutions from one 96-well plate to one square petri dish. The replicator was sterilized between each use using ethanol and a flame. Petri dishes were checked daily for lysis plaques for a maximum of 10 days.

3.3.4 Testing Resistance Type Using OtV5 Inoculation

OtV5 inoculum was prepared prior to the start of the experiment and stored at −80°C in 10% DMSO (final concentration) and inoculations were performed from the frozen stocks. The experiment did not include a co-evolving virus which allowed me to measure host evolution relative to the ancestral virus. After 32 weeks of evolution, each population was inoculated with a suspension of OtV5 particles to test whether it was susceptible or resistant to viral lysis. Samples were tested by inoculating 1 mL cell culture at a density of 10^5 with 10 μL OtV5 in 48-well plates with three replicates for each sample. Negative controls that were not inoculated with OtV5

were used as a comparison of cell growth. Cell density was measured using a FACSCanto flow cytometer 3 days after inoculation. Samples were run on 96-well plates by counting the total number of cells in 10 μL with a flow rate of 2.0 μL per second.

Data were analyzed with linear mixed effects models using the statistical packages lme4 (Bates *et al.*, 2014) and lmerTest (Kuznetsova *et al.*, 2015) in R (version 3.2.0) to identify differences in cell densities after OtV5 inoculation compared to controls that were not inoculated. Selection environment, resistance type and treatment (inoculated or not inoculated) were set as fixed effects with population as a random effect. Post hoc Tukey tests were performed using lsmeans to confirm where significant differences occurred within the different effects.

3.3.5 Population Growth Rates, Cell Size and Cell Chlorophyll Content after Evolution

At the end of the evolution experiment, I quantified evolutionary responses by measuring average cell division rates and by measuring average cell size and chlorophyll content for each population. All evolved populations were assayed in their selection environment and in the control environment, and all control populations were assayed in all selection environments except high temperature, since all populations in the high temperature environment went extinct and therefore there were no high temperature evolved strains. The populations that had evolved in a random environment for each transfer were only assayed in the control environment, which was not one of the environments they had been exposed to during the experiment, meaning only a correlated response (rather than a direct response) to selection could be obtained. Each population was assayed in triplicate. Due to the size of the experiment, assays were divided randomly into seven time blocks. This was factored into the statistical analysis.

Average cell division rates, which I refer to as "growth rates", are the average number of cell divisions per day over seven days, which corresponds to one transfer cycle. All populations were first maintained in their assay environment for an

acclimation period of one week, which was one full transfer cycle, prior to measuring growth rates. After acclimation, cells were counted using a FACSCanto flow cytometer before the transfer into the assay environment (to calculate the number of cells transferred into fresh media) and again after seven days of growth. Each sample was counted in triplicate. The cell counts were converted to cells per milliliter and the number of divisions per day was calculated using Equation (3.1).

$$(3.1) \quad \mu(d^{-1}) = \frac{log_2(\frac{N_t}{N_0})}{t-t_0}$$

where μ is population growth rate, and N_t and N_0 are the cell densities (cells mL^{-1}) at times t and t_0 (days), respectively. This measures the average number of cell divisions per ancestor over a single growth cycle and allows a comparison of offspring production between environments even if there are differences in the shape of the population growth curve. To avoid biases of cell divisions being dependent on the time of the cell cycle, cells were always measured at the same time of day (at the beginning of the light period when cells are in G1 phase).

Cell size was inferred from FSC (forward scatter), which was calibrated using beads of known sizes (1 μm, 3 μm and 6.6 μm). Chlorophyll fluorescence was inferred by measuring PerCP-Cy5.5 emission with excitation at 488 nm. Relative chlorophyll was analysed by taking the average chlorophyll fluorescence for all susceptible strains in the control environment and setting this to a value of 1, with chlorophyll measurements of all other strains relative to this value.

Data were analysed with linear mixed effects models. To analyse differences in growth rate, cell size and chlorophyll under different environments, selection environment, assay environment and resistance type were fixed effects and population and time block ware random effects that were treated as un-nested. An additional model was fitted to examine whether there was a difference in growth rate when populations were assayed in their selection environment or when they were assayed in a different environment, with assay as the only fixed effect and population and block set as random effects.

3.3.6 Competition Assay

To measure competitive fitness, all evolved populations were competed against a green fluorescent protein (GFP) line of *O. tauri*, which was provided by K Knox.

All evolved populations competed in the selection environment that they evolved in, and all control populations competed in the control environment as well as in each selection environment to measure plastic response. All of the random populations were competed in the control environment only. All populations, including the roGFP line, were acclimated for one week in the corresponding assay environment prior to the assay. Following acclimation, equal starting densities of 5×10^5 of each evolved population and the roGFP line were grown in 20 mL media for one week, after which cells were counted using a FACSCanto flow cytometer. GFP and non-GFP populations were distinguished by measuring fluorescein isothiocyanate A (FITC-A) emission at 519 nm with excitation at 495 nm (Appendix figure 1). Competitiveness of the evolved populations was measured relative to the roGFP line as fold change in cell density. Data were analyzed with a linear mixed effects model, with selection environment, assay environment and resistance type as fixed effects and population and assay replicate as random effects.

3.4 Results

3.4.1 Susceptibility to OtV5 after evolution

3.4.1.1 Host resistance type was maintained during evolution

After 200 generations of evolution in the selection environments, all surviving resistant (R) and resistant producer (RP) populations remained resistant to OtV5 lysis and all susceptible (S) populations remained susceptible to viral lysis in those environments (Figure 3.2). A significant interaction between selection environment, resistance type and treatment (OtV5 inoculation) affected susceptibility of *O. tauri* to OtV5 (ANOVA environment $\times$ resistance type $\times$ treatment, $F_{8,238} = 15.22$,

p<0.0001). A post hoc Tukey test showed that this was due to cell lysis of susceptible (S) populations ($t_{8,238}$=10.66, p<0.001), whereas cell density of R and RP lines did not decrease compared to controls that were not inoculated. The highest cell densities were observed in the low salt (post hoc Tukey test, $t_{8,238}$=-29.90, p<0.0001) and random (post hoc Tukey test, $t_{8,238}$=-7.54, p<0.0001) environments. The OtV5-inoculated S populations in low phosphate were the only populations where cell density fell below the starting cell density across all populations, indicating almost complete cell lysis and no cell growth for this combination of resistance type and selection environment. R and RP lines did not show decreases in cell density after inoculation with OtV5 compared to controls that were not inoculated, whereas S lines did.

Figure 3.2. Mean (± SE) cell density ml^{-1} of resistant (R), resistant producer (RP) and susceptible (S) *O. tauri* lines 3 days after OtV5 inoculation in five environments. Points represent the average of the three assay replicates for each evolved

population. There were three evolved populations of each line. Inoculated = populations inoculated with OtV5, Not inoculated = negative control populations that were grown for the same period without OtV5 inoculation. The dashed line represents the starting cell density at 100 000 cell ml^{-1}.

Resistant (R) and resistant producer (RP) populations did not show a significant difference in cell density between populations that had been inoculated with OtV5 and populations that had not (Figure 3.3). In contrast, all susceptible (S) populations inoculated with OtV5 showed a change in cell density relative to non-inoculated S populations in the same environments ($F_{2,125}$ = 66.51, p <0.0001). The largest differences in cell densities between inoculated and non-inoculated populations were observed in S populations evolved in the low salt environment, showing that whilst all populations in this environment were able to reach high densities in the absence of viruses, they were unable to grow in the presence of OtV5 (Figure 3.2). The large difference in S populations in low salt was due to the high growth rate of populations that had not been inoculated, since inoculated populations did not fall to lower densities than inoculated S populations in any other environments.
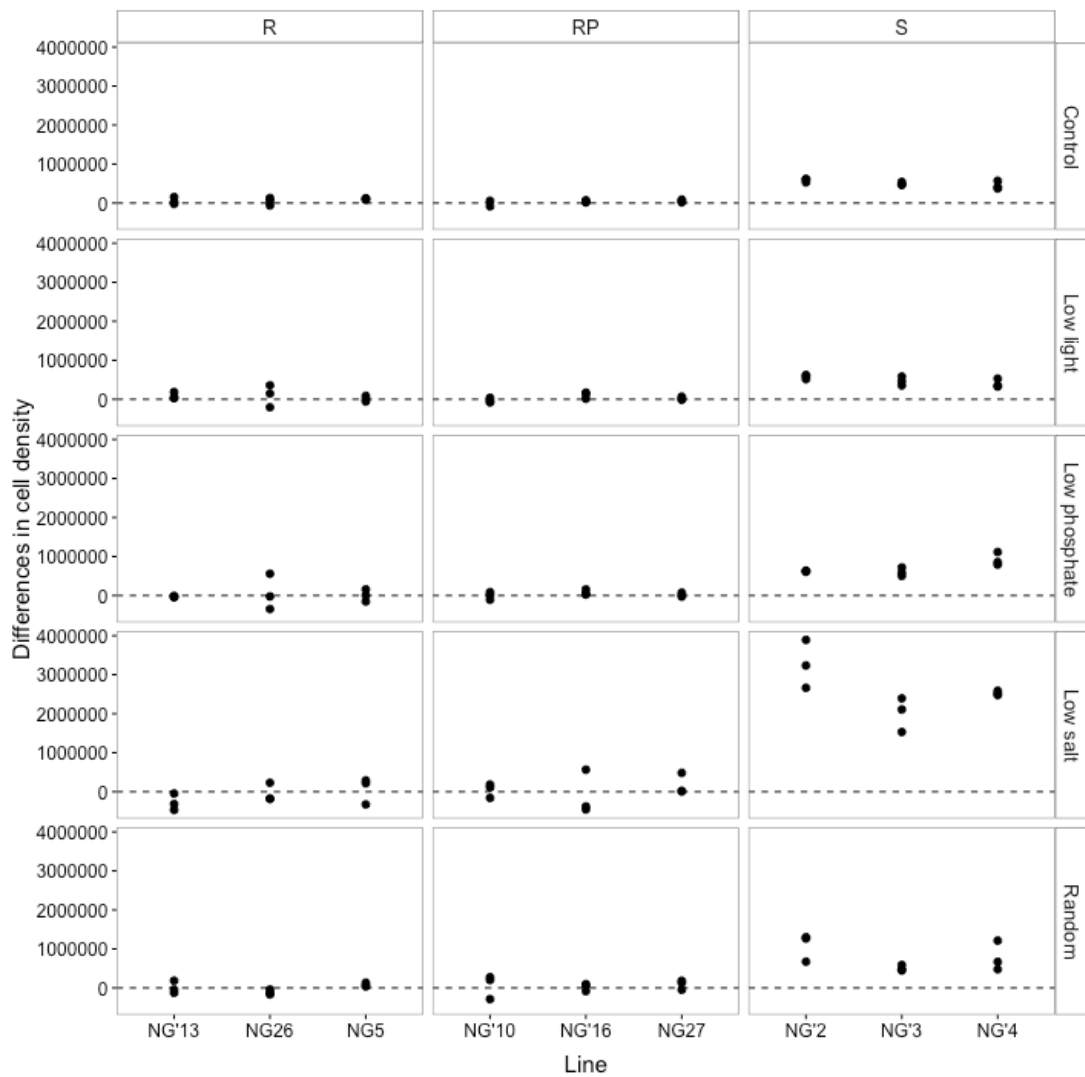
Figure 3.3. Differences in cell densities between the populations that were not inoculated with OtV5 and populations that were inoculated. The dashed line represents no difference.

3.4.1.2 OtV5-mediated lysis decreased in susceptible populations

Although susceptible (S) populations remained sensitive to viral lysis at the end of the evolution experiment, complete lysis was not observed in all populations, with a small proportion of populations able to reach numbers above the starting density of 100 000 cells ml^{-1} (Figure 3.2). This was in contrast to the beginning of the evolution experiment, when all susceptible populations fell below 100 000 cells ml^{-1} after

inoculation with OtV5, indicating near-complete lysis ($F_{1,65}$ = 21.87, p <0.0001) (Figure 3.4). The highest proportion of S cells that did not lyse was found in low salt evolved populations, suggesting that resistance mutations had been gained in this environment, despite no selection by OtV5. To eliminate the possibility that the infection dynamics had changed and that the population decline was still in process, I measured the population density 7 days after inoculation and did not observe any further decrease in population density (Appendix figure 2).
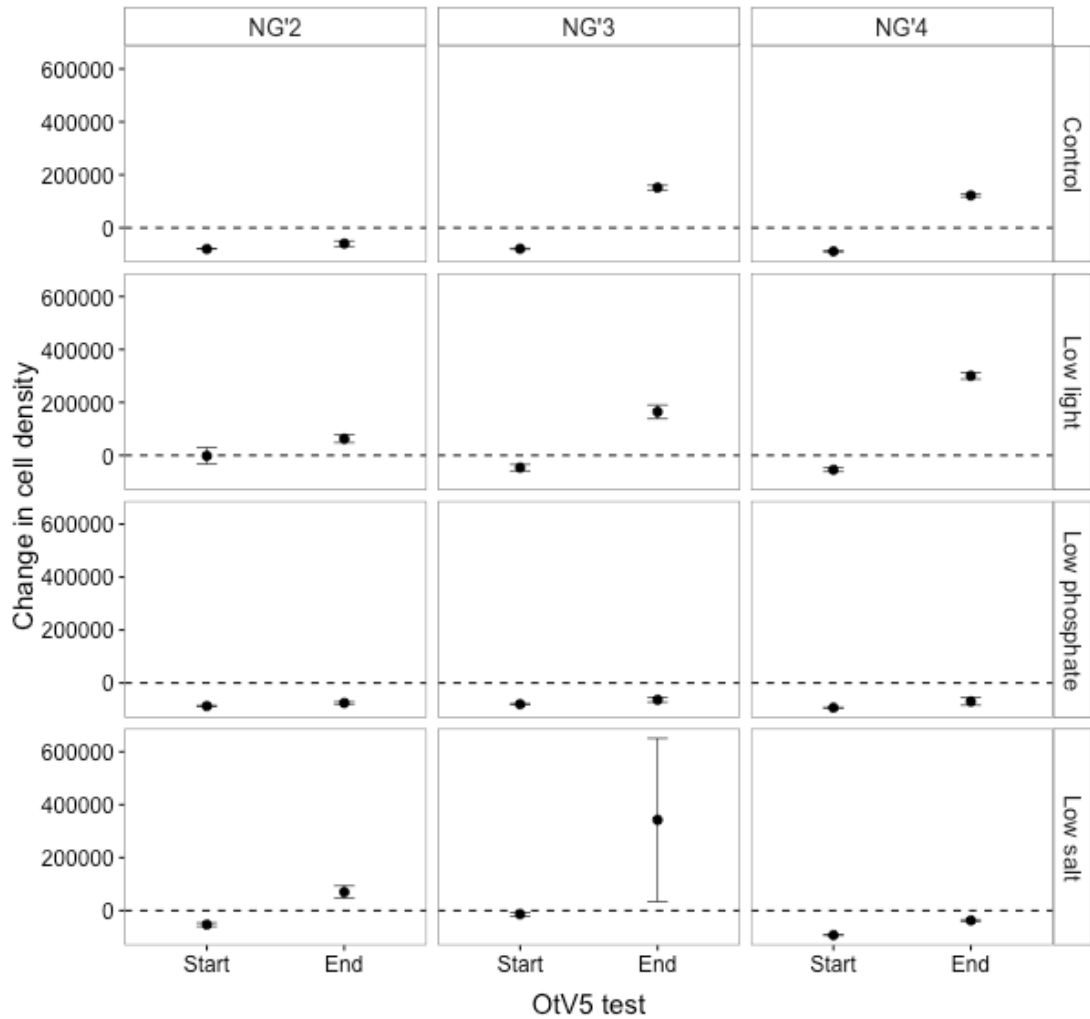
Figure 3.4. Change in cell density of the susceptible lines NG'2, NG'3 and NG'4 after OtV5 inoculation one week into the selection experiment (Start) and after 32 transfer cycles of evolution (End). The dashed line represents no change.

3.4.1.3 Resistant producers (RP) stopped producing viruses early in the evolution experiment

During the evolution experiment, resistant producer (RP) populations (NG27, NG'10 and NG'16) were tested to check that they were still producing viruses. Seven transfer cycles into the evolution experiment, all NG27 populations in all environments were still producing infectious viruses, as observed by cell lysis when their supernatant was used to inoculate the susceptible *O. tauri* strain RCC4221. In contrast, RCC4221 cultures that were inoculated with the supernatant of all populations of NG'10 and NG'16 continued growing, showing that no observable lysis had occurred. After 17 transfers in the selection environments, all RP populations in all environments had stopped producing infectious viruses (Figure 3.5), as observed by flow cytometric cell counts of RCC4221 populations inoculated with the supernatant of RP populations. When it was clear that all RP populations had stopped producing infectious viruses, frozen supernatant samples that had been collected at transfers 9, 12, 14 and 15 were tested using the plaque assay method. No plaques were observed in any samples tested, thus I concluded that all RP populations in all environments had stopped producing infectious viruses within 9 weeks of the selection experiment.
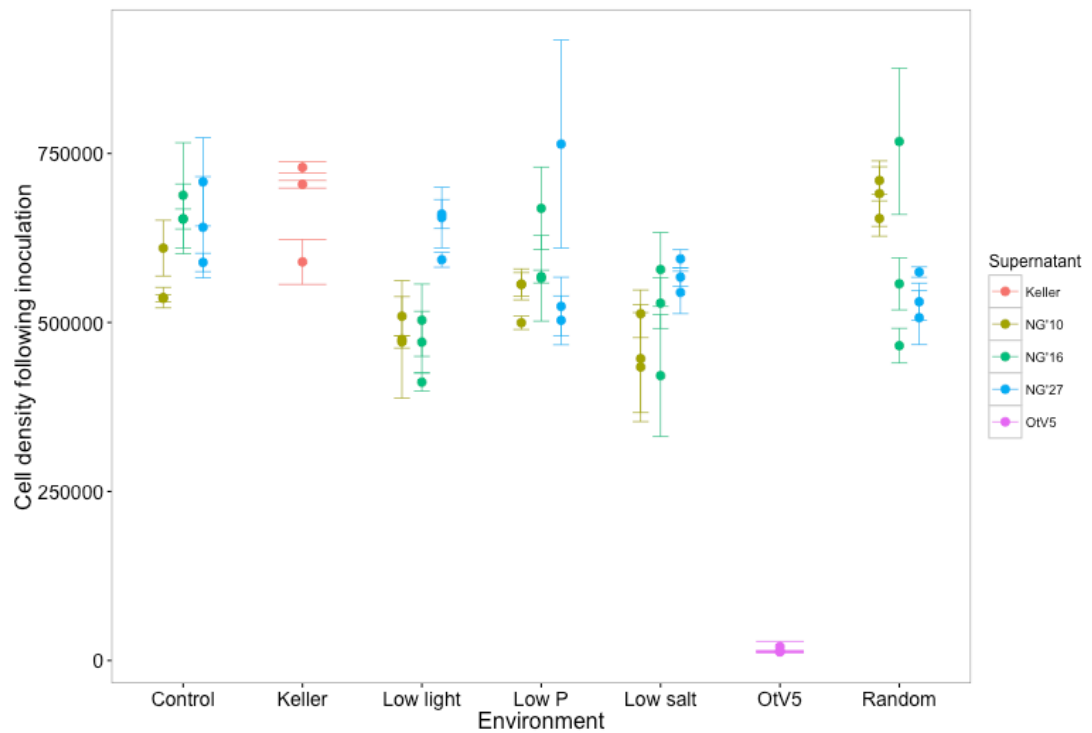
Figure 3.5. Cell density of *O. tauri* strain RCC4221 after inoculation with supernatant from three resistant producer lines NG'10, NG'16 and NG27. Inoculation with Keller media was used as a negative control and OtV5 was used as a positive control.

3.4.2 Changes in trait values after evolution

3.4.2.1 Changes in cell division rate and population persistence during the selection experiment

Here, I focus on how growth rates vary with resistance type, selection environment and the number of transfer cycles (length of time) spent in the selection environment. Growth rates of all populations were measured as the number of cell divisions per day, at four time points during the experiment (including at the beginning and end) (Figure 3.6). When comparing these time points, growth was significantly affected by environment, resistance type and time point ($p<0.0001$ for all effects). In the first transfer cycle, which measured the population growth rates at the very start of the experiment following one week of acclimation, two out of the three RP lines (NG'10 and NG'16) had increased growth rates across all environments except for low

phosphate (ANOVA effect of growth rate on cell divisions, $F_{3,5}=17.19$, p=0.046). These results are reported in Chapter 2 and Heath & Collins 2016.

After 14 transfer cycles, growth rates of all populations were approximately one division per day in the high salt, low phosphate, low light and random environments (Figure 3.6). In the control environment, growth rate varied across all S lines, even between populations of the same starting line, ranging from 0.18 to 0.87 divisions per day. The increased growth of all lines evolving in low phosphate to one division per day, which is the normal growth rate reported for *O. tauri* in phosphate-replete media, is consistent with adaptation to low phosphate in less than 100 generations. Additionally, RP lines that had been dividing more rapidly at transfer 1 were dividing at the same rate as other lines within each environment (Figure 3.6). This may be because the RP populations had stopped producing viruses and shifted to the R resistance type, thereby losing the growth advantage associated with the RP resistance type early on in this experiment. By transfer 24, all populations in the high temperature environment had gone extinct. RP populations went extinct more quickly than S and R populations, with 66% of RP lines extinct by T14 compared to 33% and 22% of S and R, respectively (Figure 3.6). At transfer 20, only three high temperature populations remained, one S (NG'4) and two R (NG'13 and NG26).
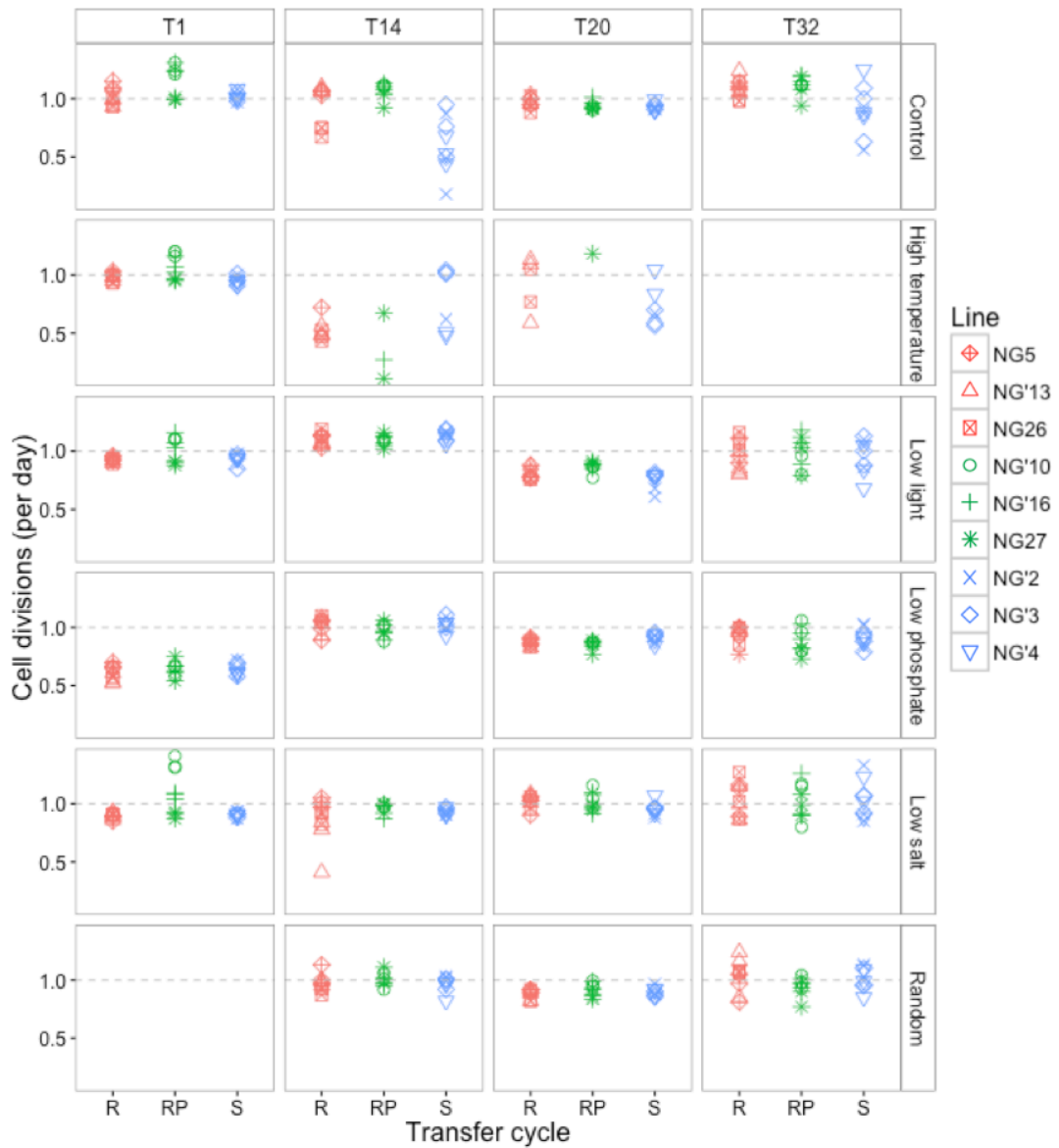
Figure 3.6. Growth rates as measured by mean cell divisions per day for each evolving population over four time points (1, 14, 20 and 32 transfer cycles). The dashed line represents one cell division per day. T1 is the growth rate following acclimation at the beginning of the experiment. There are no growth measurements for the randomised environment at T1 because lines had only been growing for one transfer cycle.

3.4.2.2 Growth rates varied with selection environment and assay environment after evolution

After approximately 200 generations of evolution in each environment, a transplant assay was performed to quantify environmental effects on population growth rate, cell size and cell chlorophyll content for each evolved population. Here I define the selection environment as the environment that the population evolved in, and the assay environment as the environment in which measurements were taken. The direct response to selection compares the growth rate of a population evolved in a given selection environment with the growth rate of a population evolved in the control environment when both are grown (separately) in that given selection environment. The effect of selection environment on the direct response to evolution was large, and driven by the direct response to selection in the low phosphate environment (ANOVA effect of selection environment on direct response, $F_{2,228} = 9.26$, $p = 0.0001$), whereas the effect of resistance type was smaller (ANOVA effect of resistance type on direct response, $F_{2,228} = 2.87$, $p = 0.06$).

An interaction between selection environment and assay environment affected growth rate, indicating that environment affected growth and the way in which selection environment affected growth differed between assay environments (ANOVA selection environment × assay environment, $F_{3,757} = 2.89$, $p = 0.03$). The fastest growth rates were seen in the evolved control populations that were assayed in low salt (Figure 3.7). Correlated response to selection is shown in Appendix figure 3. Faster growth was not due to being assayed in the same selection environment that the populations had evolved in (ANOVA effect of being assayed in selection environment on growth, $F_{1,831} = 1.70$, $p = 0.19$). Resistance type also had an effect on growth rate ($F_{2,195} = 4.21$, $p = 0.02$), with resistant (R) populations having the overall fastest cell division rates and susceptible (S) populations having the slowest cell division rates.
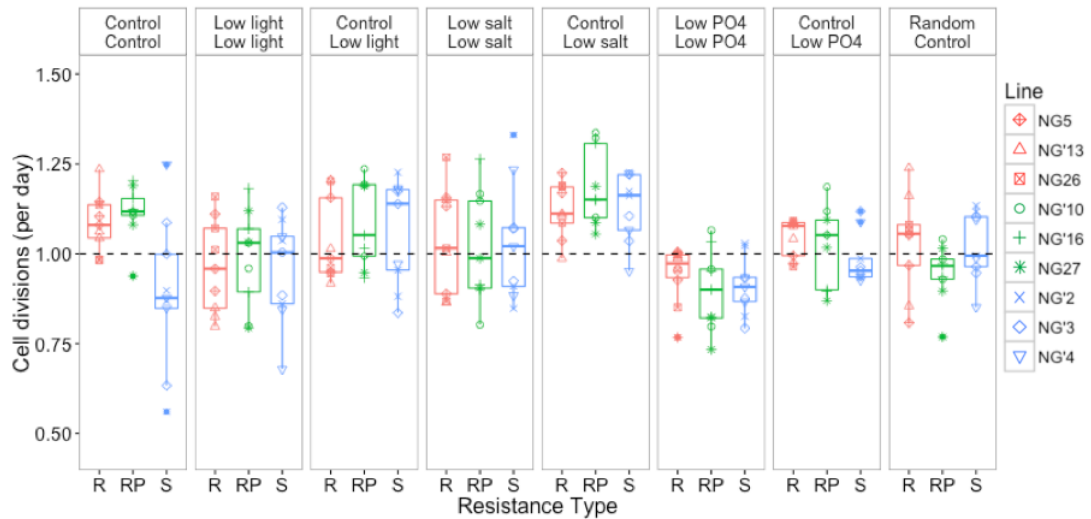
Figure 3.7. Mean *O. tauri* cell divisions per day (±SEM) showing direct response. R = resistant, RP = resistant producer, S = susceptible. Each panel represents a growth assay, with populations evolved in the selection environment (top label) and growth rates measured in the assay environment (bottom label). The dashed line indicates, for reference, one cell division per day.

3.4.2.3 Resistance type affected cell size and chlorophyll content

Cells from different resistance types had different cell sizes ($F_{2,140} = 9.49$, $p =0.0001$) (Figure 3.8, Appendix Figure 4) and this was not affected during evolution in any of the environments (ANOVA effect of selection environment on size, $F_{4,155} = 0.66$, $p = 0.62$; ANOVA effect of assay environment on size, $F_{3,735} = 1.60$, $p = 0.19$). The greatest variation in cell size between populations was observed when low light-evolved cells were assayed in low light (0.86-0.99 μm) across all three resistance types. Less variation was found in the control-evolved cells assayed in low phosphate (0.92-0.97 μm).
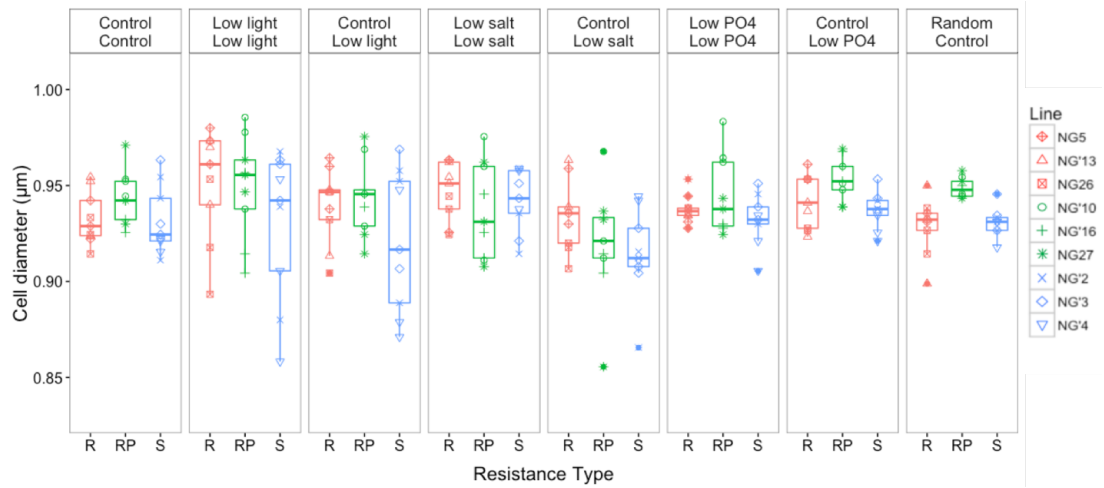
Figure 3.8. Mean cell size of *O. tauri* populations evolved and assayed under different environments showing direct response to evolution. R = resistant, RP = resistant producer, S = susceptible. Each panel represents an assay, with populations evolved in the selection environment (top label) and cell size measured in the assay environment (bottom label).

The environment in which populations were assayed had a significant effect on the relative chlorophyll content per cell volume ($F_{3,744}$ =17.83, p <0.0001). However, selection environment did not ($F_{4,168}$ = 0.90, p = 0.47). Resistance type affected chlorophyll content ($F_{2,153}$ = 8.54, p <0.0001). Susceptible populations that had been evolving in the control environment contained high amounts of chlorophyll relative to their cell size when assayed under all three selection environments (low light, low salt and low phosphate) (Figure 3.9). Appendix figure 5 shows the correlated response to selection.
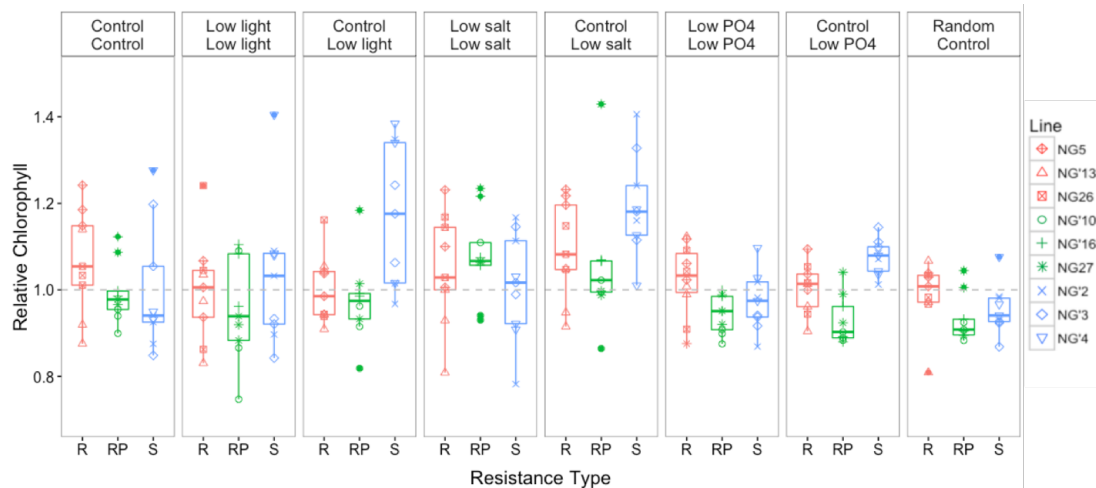
Figure 3.9. Relative chlorophyll content per cell volume of *O. tauri* populations evolved and assayed under different environments showing direct response to evolution. R = resistant, RP = resistant producer, S = susceptible. Each panel represents an assay, with populations evolved in the selection environment (top label) and mean chlorophyll content per cell measured in the assay environment (bottom label).

3.4.5 Selection and assay environments affect competitive ability of O. tauri

In addition to measuring growth rate, cell size and cell chlorophyll content, I also tested if a cost of resistance could be observed during pairwise competitions between each evolved population and a common competitor. I measured relative competitive ability by competing each population against an *O. tauri* line harbouring a GFP reporter, which allowed me to distinguish between the evolved population and the roGFP line. Both selection environment and assay environment affected competitive ability against a roGFP-labelled strain (ANOVA effect of selection environment on competitiveness, $F_{4,622} = 16.41$, p = <0.0001; ANOVA effect of assay environment on competitiveness, $F_{3,622} = 10.96$, p <0.0001). Most populations were poor competitors relative to the roGFP line (Figure 3.10). Lines evolved in low light and low salt were the best competitors. Lines that were assayed in the same environment that they had evolved in were better competitors than control lines that were assayed in the selection environments. This shows that these lines adapted to their selection environment and that growth rate is not necessarily the most appropriate measure of

adaptation in this study. Interestingly, populations in the control environment were the worst competitors, regardless of resistance type, with a 0.56 mean fold change, showing that all populations were out-competed by the roGFP line. This indicates that the control environment did in fact exert less selection on the populations than did the other environments.

Resistance type alone did not significantly affect competitive ability ($F_{2,622} = 1.22$, p = 0.30). Although competitive ability differed between resistance types, the response was not consistent across assay environments, with no single resistance type consistently being a better or poorer competitor.
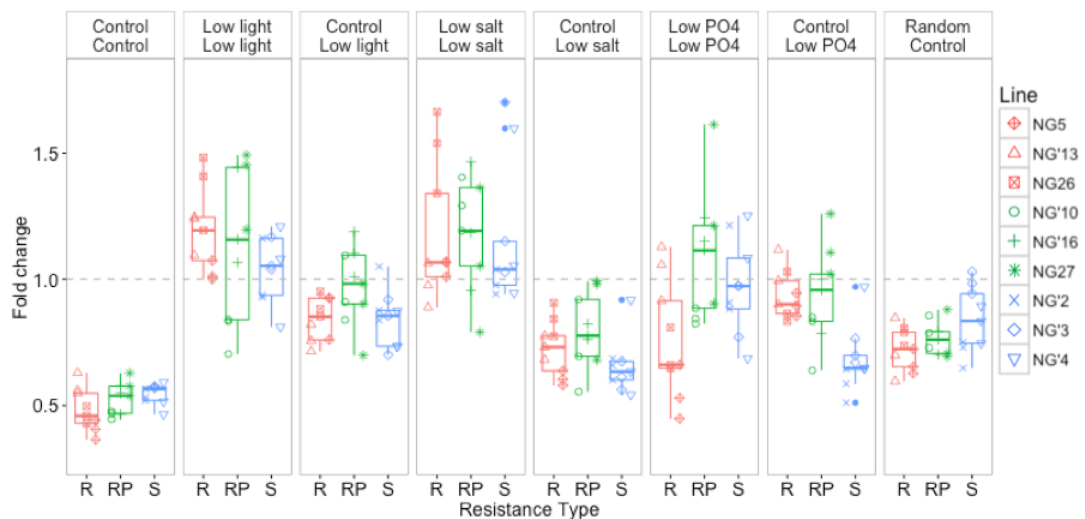
Figure 3.10. Competitive ability (as measured by fold difference in growth relative to a roGFP-modified *O. tauri* line) of evolved populations and control populations assayed in the selection environments. R = resistant, RP = resistant producer, S = susceptible. Each panel represents one assay, with populations evolved in the selection environment (top label) and competitiveness measured in the assay environment (bottom label). The dashed line represents no change (i.e. equal proportions of roGFP and competitor populations).

3.5 Discussion

I examined whether cost of resistance varied with the abiotic environment in which *O. tauri* populations evolved. A cost of resistance can manifest in different ways depending on the interaction between host and virus and on the way in which resistance is acquired (e.g. entry of the virus into the cell, and ability of the virus to replicate within the cell and cause lysis). This means that it is often difficult to detect a cost of resistance, so I measured three host responses: ability to maintain resistance, population growth rate and competitive ability.

3.5.1 Susceptibility to OtV5 Did Not Change after Evolution

After evolution in a new environment, OtV5 was still able to lyse susceptible (S) *O. tauri* populations under all environmental conditions tested, whereas R and RP populations remained resistant under all environments, despite the absence of selection pressure for viral resistance (Figure 3.2). Resistance to pathogens often comes at a fitness cost, such that a proportion of susceptible individuals remain in the population, thereby allowing viruses to persist (Lenski, 1988a). If resistance does carry a fitness cost, populations should revert to susceptibility over time, in the prolonged absence of viruses, even if that cost is low, because susceptible cells have a fitness advantage in the absence of viruses (Meyer *et al.*, 2010). My study indicated that if there is a cost to simply maintaining resistance in *O. tauri*, it is small.

It is possible that there is a genetic constraint preventing the loss of resistance, making the transition from resistant to susceptible phenotypes rare even if resistance is costly. This is consistent with recent studies showing that the resistance mechanism in *O. tauri* is an intracellular response (Thomas *et al.*, 2011) and probably also involves rearrangements of chromosome 19 (Yau *et al.*, 2016). The presence of a genetic constraint on losing resistance would favour compensatory mutations that lead to alleles being selected that reduce the cost of resistance (Lenski, 1988b; Björkman *et al.*, 2000). Studies evolving *E. coli* in the absence of bacteriophage found that the cost of resistance to the T4 bacteriophage decreased after 400 generations due to compensatory adaptations (Lenski, 1988b). A second

possibility is that the cost of resistance to one strain of OtV means increased susceptibility to other virus strains. For example, cyanobacteria can rapidly evolve viral resistance when coevolving with viruses, however increased resistance to one virus can lead to a narrower resistance range thereby making cells more susceptible to other virus strains (Avrani *et al.*, 2011; Marston *et al.*, 2012). *O. tauri*-virus interactions can be complex with some OtVs being very specific to host *O. tauri* strains while others are generalists that can infect many strains (Clerissi *et al.*, 2012; Bellec *et al.*, 2014). My experiment focused only on OtV5 and did not examine evolution of host resistance range.

At the end of the evolution experiment, OtV5 lysed susceptible (S) populations in all environments, but the extent of lysis differed between environments (Figures 3.2 & 3.3). This could be because one or more resistance mutations had appeared and risen to a detectable frequency in some populations. It is unclear whether incomplete lysis was due to some resistant cells evolving in the susceptible populations, or whether susceptible populations had evolved to make virus entry harder but still possible. Inoculations were performed from frozen stocks, thus OtV5 was not coevolving with the host, enabling me to measure evolution in the *O. tauri* populations relative to the ancestral virus population. Physiological changes in susceptible populations arising as an adaptive response to abiotic environmental change did not prevent viral lysis, indicating that viral adsorption was not completely inhibited. This was even evident in the control populations, suggesting that although these populations did not experience a change in environment, they may have evolved changes in cell surface proteins, since they were still evolving for the full length of the experiment. However, the biotic environment plays a larger role in resistance acquisition, since resistance to viruses is selected for by the virus (Luria and Delbrück, 1943). Chemostat experiments to monitor population dynamics in *Chlorella* and *Paramecium bursaria Chlorella* Virus 1 (PBCV-1) showed that control populations maintained in the absence of viruses did not evolve resistance to the ancestor virus, suggesting that resistance arises from host-virus interactions (Frickel *et al.*, 2016). In contrast, sensitive *E. coli* cells evolved complete resistance to λ phage infection and resistant cells increased susceptibility to T6* infection after 45,000 generations in the

absence of phage (Meyer *et al.*, 2010). In my evolution experiment, low phosphate was the only environment in which the cell numbers of all lines fell below the starting cell density (Figure 3.2), suggesting that this environment either affected the infectivity of OtV5 directly or the cells' response to infection. Other studies report the opposite, with reduced virus infection of algae under low phosphate, possibly due to the requirement of phosphate for viral replication (Bratbak *et al.*, 1993; Bellec, Grimsley, Derelle, *et al.*, 2010; Maat *et al.*, 2014). Though phosphate levels were low in my experiment, they were sufficient for population growth to be positive, and were higher than found in the Mediterranean Sea (Karafistan *et al.*, 2002). Conflicting results highlight the complexity of host-virus interactions in different study systems as well as different growth conditions.

There was a selection pressure against viral production but not on host resistance across all RP lines in all environments. Similarly, Yau *et al.* (2016) reported that over a two-year period RP populations maintained under standard laboratory conditions stopped producing viruses. If RP populations are indeed made up of a majority of resistant cells with a small proportion of susceptible cells arising that lyse upon OtV5 infection, thus maintaining the production of viruses in the media, then I would expect resistance to be selected for in the presence of viruses. Resistance in *O. tauri* is expected to be caused by over-expression of glycosyltransferase genes on chromosome 19 (Yau *et al.*, 2016). In my study, the selection environment did not affect the time it took for a selective sweep of resistance to occur in the RP lines, supporting the conclusions that there was little or no selection against resistance, that there is a genetic constraint on losing resistance, or that compensatory mutations enabled resistance to be maintained.

3.5.2 Resistance Type and Environment Affect Evolutionary Response of O. tauri to Environmental Change

I did not observe a growth cost of *O. tauri* being resistant to viral lysis, since R populations had the fastest growth on average overall whereas S populations had the slowest growth. Data on the growth effects of resistance in marine phytoplankton are rare. A 20% reduction in growth was reported in the ubiquitous cyanobacterium

Synechococcus (Lennon *et al.*, 2007), however it is unknown whether viral resistance generally carries a growth cost in eukaryotic algae. Even with no or minimal costs of resistance, the chromosomal rearrangement associated with resistance in *O. tauri* means that the different resistance types could have different genetic backgrounds. Therefore, evolution could take different trajectories in hosts with different resistance types due to epistatic interactions between resistance and adaptive changes. For example, trade-off shape varied in response to environmental change and physiological changes of bacteriophage resistant *E. coli*, leading to variation in sensitivity to environmental change across different strains (Jessup and Bohannan, 2008). In my study, when considering the direct response to evolution (which compares the growth rate of the evolved population in its selection environment with the plastic response of the control line in that selection environment), resistance type did not drive direct response. This indicates that the growth response of the three resistance types was similar within environments. If there is an effect of genetic background being introduced by resistance, it is not evident at the level of growth rate under these conditions.

Selection environment affected population growth, with populations evolved in the control environment having the highest growth rates in all assay environments (Figure 3.7). The decrease in growth in response to the selection environments is consistent with them being of lower quality than the control environment, by design, so that selection was stronger in the non-control environments. Variation in the direct response to evolution was explained by selection environment. Populations evolved in low phosphate had the lowest growth, which is expected when cells are nutrient limited. Interestingly, populations that had evolved in the control environment grew more rapidly in low phosphate than populations that had evolved in low phosphate. I suggest this may be because populations that had been evolving in the control environment had enough phosphate reserves within the cell to grow normally for a short period, since growth was only assayed over seven days. Overall, growth rates of populations evolved in the control environment were greater when assayed in the selection environments than the populations that had evolved in those environments, showing that increased growth could be initiated as a stress response, and that cells

in the control environment (which was nutrient-replete, and at the optimal temperature and usual salinity for these lines of *O. tauri*) were in better condition overall. The extent of a cost of resistance can be highly dependent on environment (Meaden *et al.*, 2015). For example, cost of resistance differs when fitness of *E. coli* is measured under different nutrient resources and concentrations (Bohannan *et al.*, 2002, 2013). I show here that growth rate measurements may not be sensitive enough to detect very small differences between populations conferring a cost of resistance in *O. tauri*, as has also been observed in short term experiments using a single (Thomas *et al.*, 2011) and multiple environments (Chapter 2; Heath & Collins 2016). Studies in bacteria also found that resistant strains grew at the same rate as susceptible strains (Lenski, 1988a, 1988b). My results indicate that, regardless of resistance type, *O. tauri* is able to adapt to environmental change including low light, low salt and low phosphate. However, all populations in the high temperature environment went extinct, despite the modest (2°C) increase, suggesting that although *O. tauri* can tolerate and grow at higher temperatures over the short-term, sustained temperature increases may exert stronger selection than predicted from short-term studies. It is not possible to infer as of yet whether resistance affects growth rate in natural habitats or whether a cost of resistance is instead associated with trade-offs that are not related to the abiotic environment, such as resistance to other viral strains.

In contrast to cell division rates, resistance type affected cell size and chlorophyll content, but selection environment did not. Cells in RP populations were sometimes larger in size and S populations were slightly smaller. Often, small size is associated with a response to nutrient limitation, increased temperature and light limitation in phytoplankton (Geider *et al.*, 1986; Atkinson *et al.*, 2003; Finkel *et al.*, 2010; Morán *et al.*, 2010; Peter and Sommer, 2015), however all lines in this study showed slightly increased cell size in low phosphate. An increased cell volume has also been observed in coccolithophores in response to phosphate limitation suggesting the adaptive strategy is to reduce phosphorous requirements rather than to increase surface area to volume ratio (Šupraha *et al.*, 2015).

Following evolution, RP populations had less chlorophyll in most environments, however overall there was substantial variation in chlorophyll content, especially in S populations. When assayed in the control environment, populations that had evolved in low light, low salt, low phosphate and the random environment had lower chlorophyll than did control populations assayed in these same environments. The response of populations evolved in the control environment increasing their relative chlorophyll content when assayed in low light is consistent with responses to light limitation in other green algae (Ryther and Menzel, 1959; Wozniak *et al.*, 1989; Renk and Ochocki, 1998). Here, I show that response of chlorophyll content to environmental change is variable, both with environment and with resistance type. Previous studies in marine microalgae have reported reduced chlorophyll content under nutrient limitation (Riemann *et al.*, 1989) and higher chlorophyll content under some optimal salinities (McLachlan, 1961; Sigaud and Aidar, 1993).

3.5.3 Resistance Type Did Not Affect Competitive Ability Regardless of Environment

Reduced competitive ability has often been observed as one of the main restrictions for resistance spreading through a population, however resistance type did not affect the competitive ability of evolved populations in my experiment. I found that environment did affect competitive ability, and similarly in bacteria, the environment that populations evolve in, such as the limiting sugar source or spatial heterogeneity, can affect competitive ability, both with and without coevolving phage (Brockhurst *et al.*, 2004; Jessup and Bohannan, 2008; Bohannan *et al.*, 2013). Other studies have reported a trade-off between competitive ability and resistance, whereas here I found no evidence for reduced resistance with increased competitive ability. The nature of a cost of resistance will depend on the genetic or physiological changes to the cell. For example, *E. coli* mutants showed high variability in competitiveness which was associated with resistance strategy, with cross-resistance to phage T7 significantly decreasing competitive fitness by approximately 3-fold (Lenski, 1988a). In contrast, competitions with cyanobacteria showed that total resistance (the total number of viruses to which a host strain was resistant) did not affect competitive ability

(Lennon *et al.*, 2007). These examples reveal that the magnitude of the reduced competitiveness trade-off can depend on the specific resistance strategy.

Evolved populations in the non-control environments were better competitors than control populations that had been exposed to the selection environments for the first time (plastic response), indicating that all lines had adapted to their selection environment. Thus, I suggest growth rate is not the most appropriate measure of adaptation in *O. tauri*, since the plastic response was to increase population growth rates, and the evolutionary response was to reverse this plastic increase in growth rates, and this strategy was associated with an increase in competitive fitness. Similar results have been reported previously in *Ostreococcus* spp. where populations with high growth rates in monoculture were poorer competitors than those with lower growth rates in monoculture (Schaum and Collins, 2014).

3.6 Conclusions

Here, I show that there was no detectable cost of resistance to OtV5 as measured by growth rate or competitive ability for *O. tauri* evolved in several different environments, and that resistance to viruses did not affect adaptation to environmental change. I found no reversion of R or RP populations to S as tested by exposure to OtV5, whereas lysis occurred in all S populations. Additionally, all RP lines stopped producing viruses within nine weeks of the experiment but remained resistant to lysis. This suggests that a shift from susceptibility to resistance is more common than a shift from resistance to susceptibility, regardless of selection environment, at least for the range of environments used here. My experiment shows that the conditions under which a cost of resistance may occur or affect adaptation in *O. tauri* are not clear in the laboratory. More work is needed to understand the factors that affect host–virus interactions in the marine environment to better understand evolutionary and ecological responses of marine eukaryotic microalgae to environment change.

4. Does phosphate availability affect virus production in resistant producer *O. tauri* populations?

4.1 Abstract

Some laboratory cultures of resistant *Ostreococcus tauri* can coexist with infectious viruses (OtVs). It is currently proposed that these viruses arise through the lysis of a minority of susceptible (S) cells in a mainly resistant (R) population. These cultures are referred to as "resistant producers" (RP). Virus production in RP cultures is unstable: eventually viruses are lost and all cells within the population remain resistant. I performed experiments to examine whether phosphate limitation affected the number of viruses produced and the length of time it took for RP lines to stop producing viruses. Phosphate is known to affect marine virus production and abundance in experiments and in natural marine environments. I grew three RP lines in high, medium and low phosphate for 30 weeks. I found that (1) virus production stopped in some populations in all three environments; (2) phosphate concentration significantly affected the length of time taken for lines to completely stop producing; and (3) virus abundance fluctuated over time in RP lines. These results show that there is selection pressure for RP lines to shift to the R resistance type and that phosphate limitation can affect virus abundance in some cases. My results also provide support for the hypothesis that RP populations consist of both S and R cells and that the dynamics of these populations are complex, possibly with cycles of virus infection and lysis of S cells.

4.2 Introduction

4.2.1 The enigma of the RP resistance type in *O. tauri*

Ostreococcus tauri is a unicellular marine picoeukaryote that inhabits Mediterranean lagoons and is sensitive to virus infection by *Ostreococcus tauri* viruses (OtVs). Susceptible (S) *O. tauri* cells lyse upon infection by OtVs, whereas resistant (R) cells appear to grow normally (Thomas *et al.*, 2011). In 2011, Thomas *et al.* reported the

discovery of the resistant producer (RP) resistance type in *O. tauri* (Thomas *et al.*, 2011). These were cultures in which infectious viruses were detected within host *O. tauri* populations that were resistant to OtV5 inoculation. At that point, it was thought that these viruses were being produced by resistant *O. tauri* cells and being released from the cells without causing lysis, so these populations of cells were accordingly named "resistant producers". Thomas *et al.* analysed electron micrographs of RP cultures which contained OtV viruses in association with host *O. tauri* cells (Figure 4.1). They rarely observed virus particles inside cells of these cultures (Figure 4.1A), in contrast to micrographs of infected susceptible cultures that contained many viruses. Based on this, they concluded that virus particles leave host cells in vesicles via budding (Figure 4.1B).
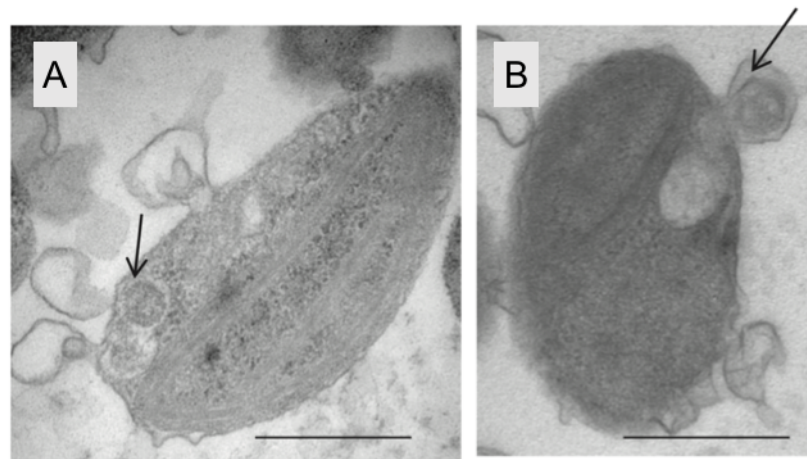
Figure 4.1. Electron micrographs of resistant producer *O. tauri* cells, taken from Thomas *et al.*, 2011. Black arrows indicate OtV5 particles.

However, in 2016, new data on the RP lines emerged from Yau *et al.* who proposed a new hypothesis that there is not an independent RP cell type that releases viruses by budding, but instead that RP *O. tauri* cultures consist of both resistant and susceptible cells, and that RP populations maintain a virus population from the lysis of susceptible cells (Yau *et al.*, 2016). The authors described very few cells showing visible virus particles in their cytoplasm (Figure 4.2A) and cell lysis (Figures 4.2B and 4.2C).
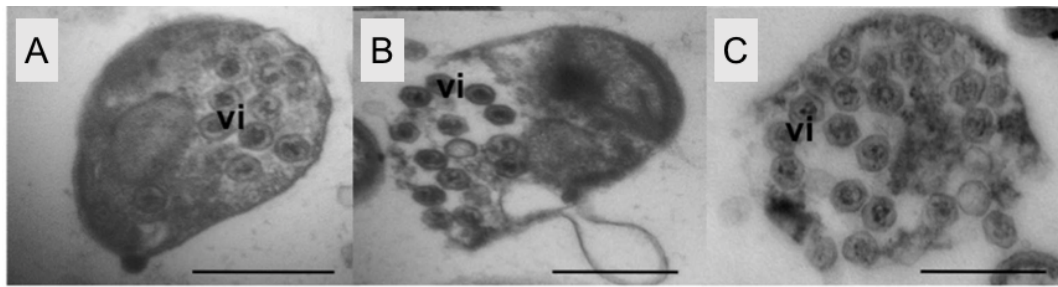
Figure 4.2. Electron micrographs of resistant producer *O. tauri* cells, taken from Yau *et al.*, 2016. *O. tauri* cells are shown with intracellular viruses (A) and undergoing lysis (B & C).

My selection experiment (Chapter 3, Heath *et al.* 2017) and the study by Yau *et al.* (Yau *et al.*, 2016), both demonstrated that RP lines are not stable and eventually stop producing viruses and become R. No shift from RP to S has ever been observed. This was important, because it changed the way that I viewed the study system for my thesis. First, I observed the eventual loss of virus production in RP cultures (Chapter 3), which is expected if RP populations are initially a mix of S and R cells. Second, I shifted my focus to understand why the RP variant is maintained and then why it always disappears with the loss of viral production. Understanding the relationships between S, R and RP host populations and viruses is necessary for predicting whether the effects of environmental change on *O. tauri* will be the same across resistance types.

4.2.2 The effect of phosphate limitation on marine viruses

Several studies have investigated the direct and indirect effects of nutrient limitation on marine viruses, including phycodnaviruses and cyanophages. Most studies have found that low phosphate conditions have inhibiting effects on virus production. The most likely reason for this is because phosphate-rich nucleic acids are needed to replicate the virus genome, therefore if the host is starved, there will be no available material for this. For example, in experiments, phosphate limitation led to a decrease in *Emiliania huxleyi* viruses (Bratbak *et al.*, 1993) and an 80% reduction in burst size of *Micromonas pusilla* viruses (Maat *et al.*, 2014), and in natural seawater samples,

OtVs are more abundant in phosphate-rich waters (Bellec *et al.*, 2010). Similarly, studies examining an increase in phosphate availability have found increased virus production and abundance, however this is likely to be an indirect effect due to the enhanced growth of their prokaryotic hosts (Tuomi *et al.*, 1995; Sandaa *et al.*, 2009; Motegi *et al.*, 2015). Phosphate availability has also been observed to influence prophage induction and lysogeny in some marine bacteria (Wilson and Mann, 1997; Wilson *et al.*, 1998; Williamson *et al.*, 2002; McDaniel and Paul, 2005). In contrast, nutrient limitation (nitrogen and phosphate) of the haptophyte *Phaeocystis pouchetii* did not inhibit viral production, although the reasons for this were unclear (Bratbak *et al.*, 1998).

The importance of a virus replicating in a phosphate-rich host is consistent with the genes encoded by many viruses, which can enhance phosphate uptake. For example, some cyanophages contain copies of *pstS*, a gene for a high-affinity phosphate-binding protein (Sullivan *et al.*, 2005); a putative phosphate permease has been described in *Emiliania huxleyi* virus EhV86 (Wilson *et al.*, 2005); and several phycodnaviruses encode *pho4* genes, from the PHO4 superfamily that encode phosphate transporters (Monier *et al.*, 2012). *pho4* is also encoded by OtV-2, a virus that infects a strain of *Ostreococcus* that was isolated from warm, oligotrophic waters that are often phosphate-limited (Weynberg *et al.*, 2011). In contrast, OtV-1 and OtV5 do not encode *pho4* and their host, *O. tauri*, inhabits nutrient-rich coastal waters and lagoons, which may mean that OtV replication within hosts in this environment is rarely phosphate limited.

The observations described above demonstrate that phosphate is important for marine virus production and that limitation of this nutrient could have important ecological consequences by affecting virus abundance, which could in turn affect the infection and lysis rates of hosts and thus host population sizes. Although the environment can have direct effects on viruses through increased decay or physical damage to the particle, effects of phosphate are much more likely to have indirect effects since viruses cannot take up or process nutrients directly. One possibility for the high phosphate requirement of viruses is that viruses have a higher

phosphate: nitrate ratio compared to cellular organisms because of their higher nucleic acid: protein content (Bratbak *et al.*, 1993).

4.2.3 Does phosphate limitation drive virus extinction in RP populations?

I showed in Chapter 3 that all of my RP lines stopped producing viruses within a few weeks, regardless of their selection environment (Heath *et al.*, 2017). Yau *et al.* (2016) also observed virus loss in their RP lines, however the timing of this loss was variable and some of their RP lines continued to produce viruses over many months. These observations show that there is selection pressure for virus production to stop in RP lines in most environments, but the strength of selection probably varies between environments. I suggest that this is because of the selection pressure for resistance, meaning S cells become lost. Alternatively, if there is an independent RP resistance type, tolerating chronic virus infection and avoiding cell lysis while producing viruses could be costly and therefore strongly selected against.

In this chapter, I performed an experiment to investigate how quickly RP populations stop producing infectious viruses and whether environmental drivers can affect virus production. I chose to grow RP lines under different phosphate concentrations, since phosphate has previously been found to affect virus production. Additionally, the *O. tauri* host and OtVs have mainly been isolated from Mediterranean lagoons, where they experience large nutrient gradients (Bellec *et al.*, 2009) and the Mediterranean Sea is mainly phosphorous limited (Lazzari *et al.*, 2016). I hypothesise that under lower phosphate concentrations, RP populations will produce fewer viruses and therefore the shift away from RP will be quicker.

4.2 Methods

4.3.1 Lines and culturing conditions

Five resistant producer (RP) *O. tauri* lines (NG1, NG14, NG'16, NG25 and NG27, provided by N. Grimsley) were grown in triplicate in Keller medium that contained full (10 μM), half (5 μM) or a quarter (2.5 μM) of the usual phosphate concentration

(Keller *et al.*, 1987). For simplicity, these environments will be referred to as high, medium and low phosphate. The cultures were not axenic. Seawater was made using Instant Ocean supplemented with Keller medium and f/2 vitamins and 0.22 μm filter sterilised. Cultures were incubated at 18°C in a 14:10 hour light: dark cycle. Cultures were transferred to fresh medium when they reached a density of approximately 10^6 cells per ml, which corresponded to every 7-10 days. The experiment was performed for 20 transfers, which corresponded to approximately 140 generations.

4.3.2 Testing for viral production

4.3.2.1 Virus production before starting the experiment

At the start of the experiment, all RP lines were tested for the presence of viruses using three techniques: a liquid lysis technique, a plaque assay technique and PCR. The liquid lysis assay and plaque assay tests both tested for virus infectivity, whereas PCR tested for the presence of OtV5 DNA.

To obtain viruses, 1 ml of each RP culture (approximately 10^6 cells) was centrifuged at 1000 × g for 20 minutes to form a pellet of cells separate from the media containing viruses. For the liquid lysis assay, 200 μl of each sample supernatant was transferred to 1 ml of exponentially growing susceptible *O. tauri* RCC4221 in a 48 well plate. Positive controls were prepared using a known suspension of OtV5. Negative controls were prepared that did not have the addition of any virus or RP supernatant. Plates were sealed with Parafilm and incubated at 18°C in a 14:10 hour light:dark cycle for a maximum of ten days, during which wells were checked for lysis. Wells in the negative controls were green due to growth of *O. tauri*, whereas all wells containing positive controls were clear, indicating viral lysis had occurred. If a well was clear, this indicated the presence of infectious viruses in the RP supernatant.

For the plaque assays, a lawn of susceptible cells was immobilised on agarose plates. Plates were made by preparing a suspension of 15 ml exponentially growing susceptible *O. tauri* RCC4221 added to 30 ml Keller medium and agarose (final

concentration 0.15%), which was poured into a 12 cm square petri dish and left to set in a sterile flow hood at room temperature. Ten-fold dilutions of the RP supernatants were made using 96 well plates. 1.5 μl of each serial dilution was transferred to the lawn of susceptible *O. tauri* growing on the agarose plate. Positive controls of known OtV5 and negative controls with no OtV5 or RP supernatant were also used. Plates were sealed with Parafilm and incubated at 18°C in a 14:10 hour light: dark cycle for a maximum of 10 days, after which virus plaques were counted.

PCR was used to amplify a 600 bp long fragment of the *pol*B gene of the OtV5 virus. I made OtV5-specific primers adapted from the AVS primers designed to detect viruses that infect eukaryotic algae (Chen and Suttle, 1995). An upstream primer, OTVFWD (5'-GAG GGT GCG ACT GTC CTG GAG-3') and a downstream primer, OTVREV (5'-GCG GCG TAG CGC TTT TTG GAG TAC-3') were used. The reaction was set up as follows. 2 μl of RP supernatant was added to 18 μl of reaction mixture containing 4 μl 5×GoTaq buffer, 0.4 μl 10 mM dNTP, 1 μl OTVFWD, 1 μl OTVREV, 0.1 μl GoTaq polymerase and 11.5 μl MilliQ water. A negative control contained MilliQ water and a positive control contained OtV5 DNA. The PCR cycle consisted of an initial denaturation step at 95°C (2 min), followed by 35 cycles of 95°C (30 sec), 59.6°C (30 sec), 72°C (48 sec), and a final extension at 72°C (5 min). The PCR products were viewed by loading 5 μl on a 1.5% agarose gel stained with 10 μl GelRed run at 80 V for 1 hour.

4.3.2.2 Testing RP lines for virus production during the experiment

RP populations were grown in high, medium or low phosphate for 30 weeks, corresponding to approximately 20 transfer cycles. The populations were tested for infectious virus production at each transfer during the experiment using the liquid lysis technique described above, since this method worked more reliably than the plaque assays. Five-fold dilutions of the RP supernatant were made using 96 well plates and 10 μl of each serial dilution sample was transferred to 1 ml of exponentially growing susceptible *O. tauri* RCC4221. This gave a limit of detection of 1 virus particle per 10 μl. If a well was clear after a maximum of ten days, this

indicated viral lysis and the presence of infectious viruses. Negative controls contained no RP supernatant. Estimated virus abundance was extrapolated from the number of clear wells corresponding to the dilution factor (Table 4.1). This level of resolution was appropriate for this experiment because I was only interested in the length of time it took for viral production to stop completely rather than in resolving changes in viral production when production is abundant. Populations were maintained for two transfer cycles following the transfer at which no viruses were detected. After this, I checked that the virus-free lines were resistant by inoculating them with a fresh suspension of OtV5 and comparing their growth to controls that had not been inoculated. If the population remained green, this indicated it was resistant to OtV5 lysis.

Table 4.1. The minimum number of viruses per ml corresponding to the dilution factor and the number of clear wells observed.

Number of clear wells	Dilution factor	Minimum number of viruses per ml
1	0	1
2	5	500
3	25	2 500
4	125	12 500
5	625	62 500
6	3 125	312 500
7	15 625	1 562 500
8	78 125	7 812 500

4.3.3 Statistical analysis

Data were analysed using lme4 and lmerTest in R (version 3.2.0). I used a mixed effects model to examine whether the number of viruses present in RP cultures was affected by time (as measured by transfer number) or phosphate concentration. Using transfer number accounted for initial slower growth rate in the low phosphate environments because populations were not transferred until they reached a density

of 10^6 cells per ml. I set transfer and phosphate concentration as fixed effects and line as a random effect. A second mixed effects model was used to examine whether the time it took for virus production to stop was affected by phosphate concentration or RP line. Here, phosphate concentration and RP line were fixed effects and replicate was a random effect. Finally, I examined whether phosphate concentration and line affect virus production, where production was calculated as the number of viruses produced per unit of time for each population. Environment and line were fixed effects and replicate was a random effect. To see whether phosphate affected virus production within lines, I used post-hoc mixed effects models to examine each line separately where environment was a fixed effect and replicate was a random effect.

4.4 Results

4.4.1 Testing for virus production at the start of the experiment

Before the experiment was started, I tested six RP lines for virus production using a liquid lysis assay, a plaque assay and PCR. The results are summarised in Table 4.2.

Table 4.2. Results from three tests for viruses within the media of six RP *O. tauri* lines.

RP Line	Liquid lysis assay (wells cleared)	Plaque assay (number of plaques)	PCR (band present)
NG1	Yes – incomplete lysis	0	Yes
NG'11	No	0	No
NG14	Yes – incomplete lysis	0	Yes
NG'16	Yes – incomplete lysis	1	No
NG25	Yes	4	Yes
NG27	Yes	3	Yes

Lysis was observed in five out of the six lines, as well as in the positive controls. However, lysis was incomplete in three of these lines. Plaques were identified in three out of the six lines, as well as in the positive controls. I detected OtV5 DNA in four out of the six RP lines using PCR (Figure 4.3). Out of the three techniques used, NG'11 was the only line which did not show any signs of virus production, so this line was not used for the experiment.
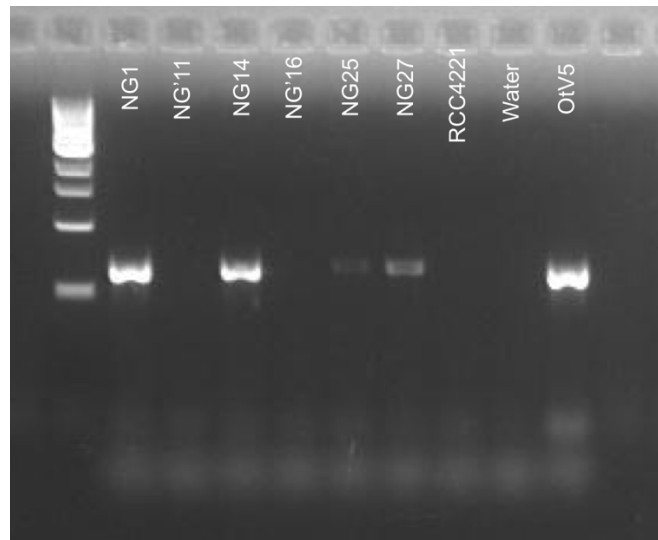
Figure 4.3. PCR products of six *O. tauri* RP lines showing bands for OtV5 amplified by OtV5-specific primers. Water and *O. tauri* strain RCC4221 were used as negative controls and OtV5 was used as a positive control.

The five RP lines that showed evidence of viruses present in the media were grown in low, medium and high phosphate. Although the liquid lysis test indicated the presence of infectious viruses for all five lines before starting the experiment, only three lines showed evidence of virus production once the experiment was started. Thus, data from three RP lines, NG'16, NG25 and NG27, were analysed and are described below.

4.4.2 Virus production in three RP lines

4.4.2.1 RP lines produced fewer viruses over time

I found that as time spent in the selection environment increased, virus production significantly decreased and was affected by phosphate concentration ($F_{2,211} = 4.99$, $p = 0.01$). This was because RP lines in the low phosphate environment stopped producing sooner. Thus, my experiment confirmed that RP lines do stop producing viruses, although the length of time that it takes for virus production to stop can vary substantially (Figure 4.4). Transfer alone significantly affected virus abundance ($F_{1,212} = 16.15$, $p < 0.0001$), whereas environment alone did not ($F_{2,211} = 0.26$, $p = 0.77$). One RP line out of the three, NG25, continued producing viruses for the duration of the experiment (20 transfers) in high and medium phosphate. In contrast, NG'16 stopped producing viruses in all three phosphate environments within six transfers. All lines remained resistant to OtV5 inoculation following the loss of virus production, as measured by cultures remaining green.
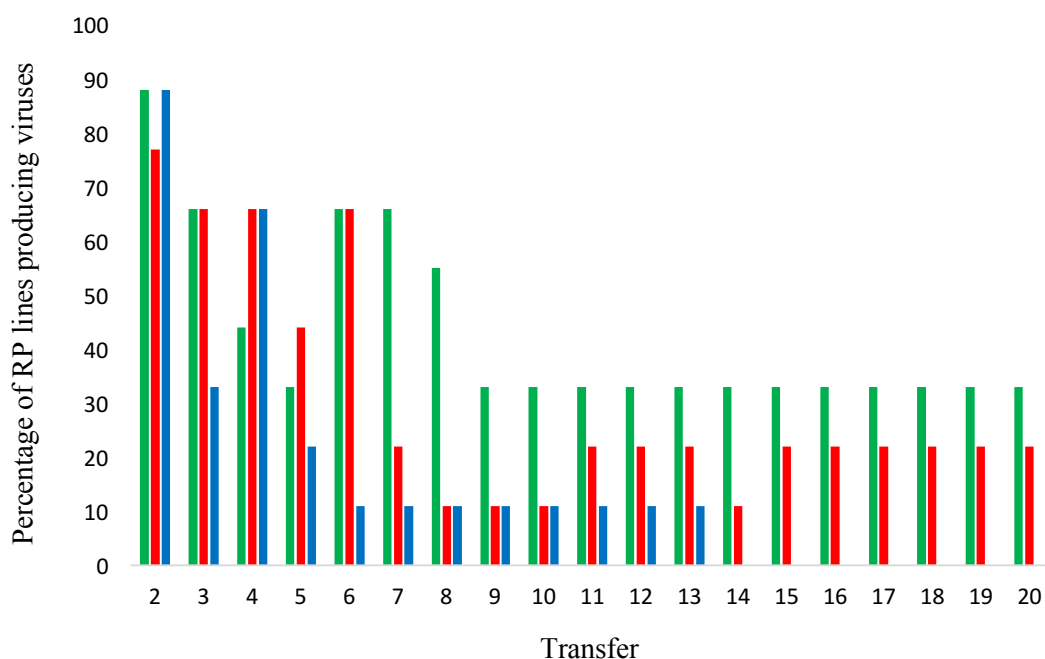
Figure 4.4. Proportion of RP lines producing viruses over the course of the experiment. High phosphate = green, medium phosphate = red, low phosphate = blue.

4.4.2.2 Virus production can fluctuate over time

Although overall virus production decreased with time, it did not decrease steadily. Rather, the abundance of viruses in the RP populations fluctuated over time across all lines and all environments, as observed by the number of clear wells measured after infection of susceptible *O. tauri* with RP supernatant (Figure 4.5). This is most apparent in NG25, which continued producing viruses for the duration of the experiment. Since I measured virus abundance at the end of the transfer cycle, this limited the power to determine the shapes of the curves. NG25 grown in the low phosphate selection environment was the only line that showed cases of a steady decline in the number of viruses present in those populations. Since I only used three RP lines, and two of these lines stopped producing viruses early in the experiment, it is unclear whether fluctuations are normal for all RP cultures. The fluctuations in virus abundance suggest that viruses are being actively produced in these populations and that the dynamics by which they are being produced may be complex. Patterns of virus production were often similar within lines and selection environments (Figure 4.5).
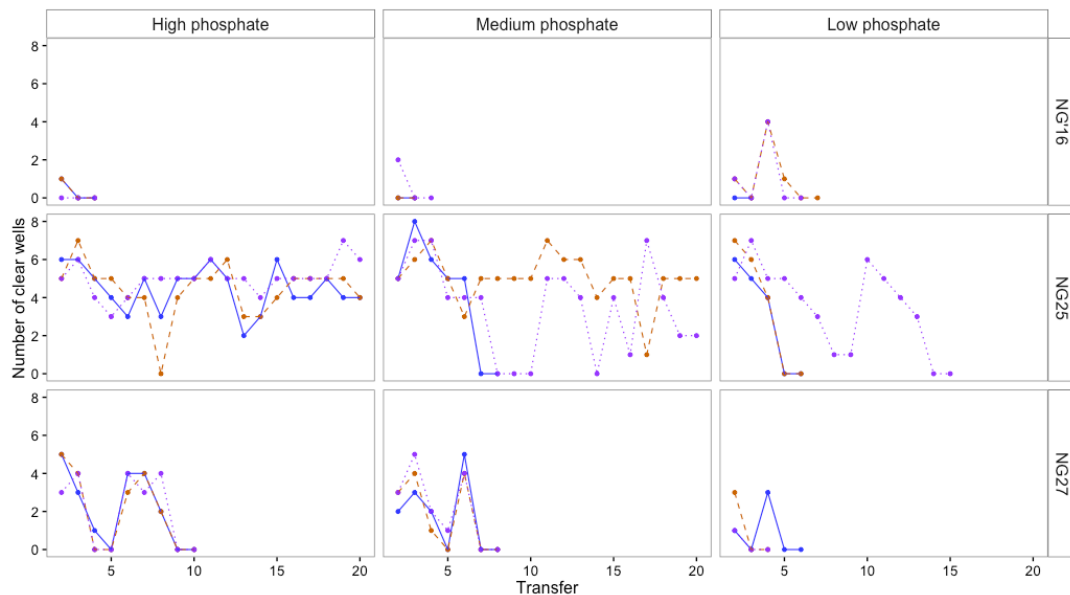
Figure 4.5. The number of clear wells observed from five-fold serial dilutions of the supernatant of three RP lines (NG'16, NG25 and NG27) that had been grown in media containing high, medium or low phosphate concentrations and used to infect susceptible *O. tauri*. Different colours and line types indicate the three independent replicates of each line.

4.4.2.3 Phosphate concentration affects the length of time taken for virus production to stop but not virus abundance

There was a significant effect of phosphate concentration on the mean time that it took for virus production to stop, which was dependent on the RP line ($F_{2,24} = 6.01$, $p = 0.002$). Overall, RP lines in the low phosphate environment stopped producing viruses before those in the high and medium phosphate environments (Figure 4.6), although NG'16 was an exception. All NG'16 lines stopped producing viruses within six transfers, however, low phosphate was the only environment in which virus production in NG'16 continued for more than two transfers and in which the most viruses were produced (Figure 4.5).

Virus abundance was not lower in RP lines grown under the low phosphate concentration ($F_{2,27} = 1.70$, $p = 0.20$), but there was a strong effect of line

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($F_{2,27} = 115.86$, $p < 0.0001$), where two of the lines stopped producing viruses after few transfers regardless of phosphate concentration, so that any effect of phosphate concentration would have been difficult to detect. The third line (NG25), which produced viruses for longer overall, showed an effect of phosphate availability on virus production in the expected direction, where decreased phosphate availability was associated with an earlier loss of virus production.
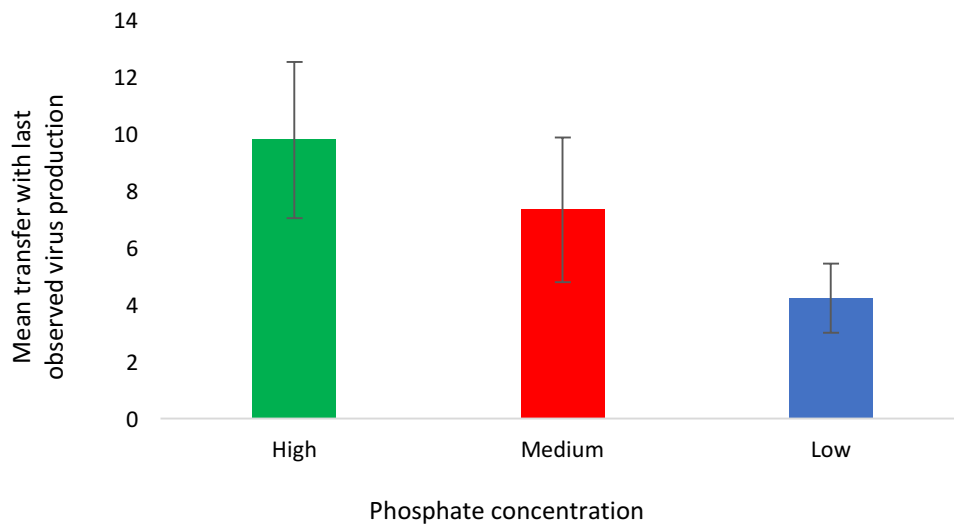
Figure 4.6. Mean (± SE) transfer number at which viruses were last identified in the media of RP lines under high, medium and low phosphate.

4.4.2.4 Lines that produce more viruses are more affected by phosphate concentration

Since there were significant effects of RP line on both the length of time taken for virus production to stop and the production of viruses, post hoc mixed effects models were used to look for differences within lines. Of the three lines, phosphate concentration significantly affected the time it took for virus production to stop in NG25 ($F_{2,6} = 8.21$, $p = 0.02$) and NG27 ($F_{2,7} = 73.50$, $p = 0.01$), and virus production in NG27 ($F = 20.24$, $p < 0.001$). This suggests that in RP *O. tauri*, phosphate has a stronger effect on lines that produce more viruses, whereas it does not have an effect on those that produce fewer, because once virus production in an RP line ceases, it

can no longer be affected by environment. Additionally, if phosphate concentration does affect the lines that stop producing viruses early, it would not be possible to detect if the loss of production is within a single transfer for both the phosphate replete and phosphate limited populations. This highlights the limitation of resolution in this experiment meaning that if viruses in the phosphate limited populations go extinct marginally sooner this will not be detected by the method that I used.

4.4.3 Using virus abundance to predict the proportions of RP or S cells in the population

The mean number of clear wells at each transfer varied between populations (Figure 4.7). The overall mean for all populations was 4.2, corresponding to approximately 12 500 viruses per ml (Table 1), although the fluctuations in virus abundance meant the range was large. To further examine the two possibilities that (1) RP phenotype cells are releasing viruses via budding (Thomas *et al.*, 2011) and (2) susceptible (S) cells are responsible for virus production through lysis (Yau *et al.*, 2016), I calculated how many RP or S cells would be needed in the population to produce the number of viruses I observed in my experiment.

I transferred the RP lines when they reached a density of $10^6 - 10^7$ cells per ml. To test the first hypothesis, assuming RP cells produce between one and three viruses per day (Thomas *et al.*, 2011), the virus population would have to have a minimum abundance of 10^6 per ml. This would correspond to a clearing of at least seven wells in the experiment. This was observed ten times out of the 215 total measurements and only in NG25 which was the only line that ever cleared seven or eight wells, mainly in the medium phosphate environment (Figure 4.5).

To test the second hypothesis, I assumed a burst size of 25 viruses per cell (Derelle *et al.*, 2008). This suggests that if the viruses are being produced from susceptible cells, S cells must account for 0.05% of the total population. This is in line with observations by Yau *et al*. (2016), and it is also low enough for the populations to remain mainly resistant to viruses overall following inoculation with a fresh OtV5 suspension, therefore supporting this hypothesis.
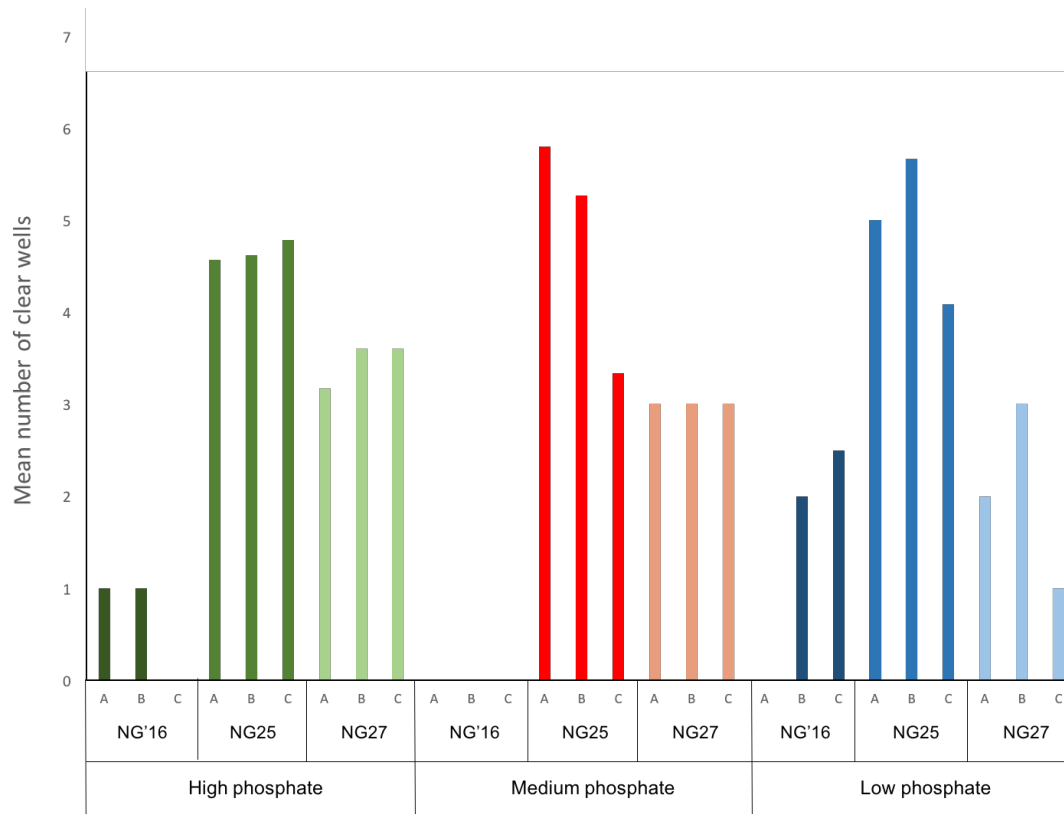
Figure 4.7. The mean number of clear wells observed for each RP population

4.5 Discussion

4.5.1 Virus production can stop in all RP lines regardless of phosphate concentration

I found that virus production eventually stopped in RP lines over time in all environments. The only case in which all three replicates of a line continued to produce viruses for the duration of the experiment was NG25 grown in high phosphate. This supports previous observations that the RP resistance type is not stable (Yau *et al.*, 2016; Heath *et al.*, 2017) even in a rich environment (i.e. the standard culturing conditions with the full phosphate concentration required for Keller medium).

These results, alongside previous findings (Yau *et al.*, 2016; Heath *et al.*, 2017), indicate that there is a selective pressure for RP lines to stop producing viruses. This

is expected if the viruses are being produced from lysis of susceptible cells, since the presence of viruses in the population imposes a selection pressure for resistance. However, I found substantial variation in the time taken for populations to stop producing. Thus, it remains unclear what other factors drive the speed of viral loss from RP populations, for example dilution to extinction. With the loss of viruses from the culture, the selection pressure for resistance (R) will be reduced. However, since resistance in *O. tauri* appears to be irreversible, at least for 200 generations, this could explain why I did not observe the populations becoming susceptible.

A decrease in virus abundance could either mean that there is a decrease in the frequency of susceptible cells, or that there is a decrease in burst size. Importantly, from the virus abundance data that I collected in this experiment, I was not able to distinguish between few susceptible cells with a high burst size and many susceptible cells with low burst size. Future work could look at which of these scenarios is causing the decrease in virus abundance, for example through the use of electron microscopy.

4.5.2 Phosphate concentration affects virus production in RP lines that continue to produce viruses

There were significant differences between lines in their responses to phosphate concentration. This is because NG'16 stopped producing viruses within a few transfers, and NG25 continued producing viruses at the end of the experiment in some populations. In the populations that continued to produce viruses for a long time, RP lines grown in high phosphate produced the most viruses and lines grown in low phosphate produced the fewest viruses. This is in line with the literature, which has generally reported a correlation between phosphate concentration and virus abundance in laboratory and mesocosm experiments (Bratbak *et al.*, 1993; Sandaa *et al.*, 2009; Maat *et al.*, 2014; Motegi *et al.*, 2015) and in natural populations (Bellec *et al.*, 2010). The link between phosphate availability and virus production is probably indirect since viruses are entirely dependent on their hosts for reproduction (Sandaa *et al.*, 2009). If the *O. tauri* host grows better in high phosphate conditions, then there will be more hosts available for viruses to infect as well as more resources

available to the virus for replication once it has infected a cell. If a host cell contains less available phosphate for the virus, burst size could be decreased under phosphate limitation. Although virus production was higher in the high phosphate environment, there was still variation in production across all environments, showing that lowered phosphate concentration did not completely inhibit viral production.

There was a significant effect of phosphate concentration on the length of time it took for RP populations to stop producing viruses; on average, lines in high phosphate produced viruses for longer and lines in low phosphate stopped producing viruses earlier. The large variation in loss of viral production highlights the complexity of this system. For example, NG'16 continued producing viruses in the low phosphate environment after the lines in high and medium phosphate had stopped. However, NG25, the only line that continued to produce viruses in some replicates for the duration of the experiment, only did so under high and medium phosphate. The length of time that it takes for virus production to stop is likely to be ecologically important because if there are viruses present within the system this will drive population dynamics and resistance in the host. The statistical power in this study is limited because I only used three RP lines, meaning that it is unclear whether this variation arises from the method or if it is an inherent property of the RP system. More lines will need to be studied in order to find out if the effect of phosphate concentration is consistent in lines that continue to produce viruses over many generations.

The standard Keller media used to grow *O. tauri* in the laboratory is already very phosphate rich and reducing it by half or one quarter in my study was still higher than that of the Mediterranean Sea, where *O. tauri* was originally isolated (Karafistan *et al.*, 2002). I did not use extreme phosphate limitation for this experiment because it was necessary for the hosts to be able to grow, however the medium and low phosphate concentrations were lower than the standard laboratory conditions that *O. tauri* is usually grown in, meaning a response to a change in phosphate concentration is expected.

4.5.3 Virus production fluctuates

I found that the number of viruses within an RP population can vary greatly and is not directly proportional to the density of host cells. This corroborates with PFGE experiments by Yau *et al.* (2016) who observed the number of copies of the OtV5 genome was not directly proportional to the host. Additionally, the number of viruses detected in the liquid lysis experiments did not decrease steadily over time, with some populations showing fluctuations in viral production over the course of the experiment. This indicates that infectious viruses were actively being produced by the hosts, and that the loss of viruses in RP cultures is not due to them being diluted to extinction. It also shows that there is not a steady rate of a shift to R. However, I cannot rule out the possibility that the fluctuations of virus abundance were due to the virus populations becoming extinct, because as the populations became smaller there would have been a stronger effect of stochastic events, or that the fluctuations were caused by differences in growth stage among the time points at which abundance was measured.

Based on the results of this experiment and modelling experiments that I will discuss in Chapter 5, I suggest that one plausible source of additional variation is that there are cycles of susceptible host cell infection and lysis, and therefore also of virus production. Contact rate between hosts and viruses is proportional to their abundances. As the number of susceptible cells decreases due to lysis, there will also be fewer viruses being released, meaning that when the proportion of susceptible cells is very low, encounter rates with viruses are low. This might then allow the susceptible cells to increase in frequency as they avoid infection, at which point encounter rates for viruses with susceptible hosts would increase again. Thus, the virus population would go extinct if all susceptible cells got infected and lysed, meaning there were no available hosts left, or if encounter rates between susceptible cells and viruses were so low that viruses were unable to infect a host. This is similar to the fluctuating selection hypothesis, which proposes that as fast-growing susceptible cells increase in abundance, and the contact rate with viruses increases, increased mortality of susceptible cells provides an opportunity for resistant cells to increase their numbers (Avrani *et al.*, 2012). However, this hypothesis relies on

susceptible cells having a growth advantage compared to resistant cells, which has rarely been observed in *O. tauri* (Thomas *et al.*, 2011; Heath and Collins, 2016; Heath *et al.*, 2017). These could be reasons for variation in the length of time it takes for virus production to stop. I investigate the dynamics of this system further in a modelling study in Chapter 5.

4.5.4 Support for susceptible cells producing viruses to explain the RP mechanism?

From my virus abundance data, I calculated how many RP or S cells would be present in the RP population to produce the numbers of viruses I observed. The virus abundances in my RP populations varied enormously, however the average number of 12 500 viruses per ml meant that at this density approximately 0.05% of the RP host population would be S. Yau *et al.* (2016) observed <0.5% of visibly infected or lysing cells in electron micrographs of RP lines. This proportion is low enough that upon re-inoculation with OtV5, RP populations would not show a significant decrease in cell abundance due to lysis. We know from several studies (Thomas *et al.*, 2011; Heath and Collins, 2016; Yau *et al.*, 2016; Heath *et al.*, 2017) that RP populations are resistant to viruses, meaning that if there are susceptible cells within the population, they would have to be at a low enough proportion for the overall population to be "resistant". Thus, I propose that there is a consistent mechanism for virus production in RP lines (S and R cells coexisting in a population), but that this mechanism gives noisy dynamics, possibly due to cycles of virus infection. My methodology is likely to have increased the noise in these data because any virus populations close to the edge of what I can detect will fall stochastically into one of the abundance categories. This means that at higher virus concentrations there are large apparent fluctuations in virus number, even if the actual fluctuations are much smaller.

4.6 Conclusions

I have shown that both the length of time that it takes for RP lines to stop producing viruses and the abundance of viruses produced by RP lines are highly variable, and that length of time to stop producing is dependent on phosphate concentration. These

findings reveal that the RP phenomenon is complex and more experimental work will need to be undertaken to elucidate exactly how viruses are being produced and what factors affect the speed at which production no longer continues. Additionally, this is likely to have ecological consequences in the oceans as I have shown that *O. tauri* hosts and OtVs can coexist temporarily, but that there is a strong selection pressure for resistance and for viruses to be driven to extinction.

5. A model of the epidemiological dynamics of OtV infection in RP lines

5. 1 Abstract

Laboratory populations of resistant *O. tauri* that coexist with infectious OtV5 viruses are called resistant producers (RP). Virus production in RP populations is unstable: viral abundance fluctuates and production usually stops, although the time it takes for viruses to be lost from the population is highly variable. Two hypotheses have been proposed to explain the origin and maintenance of the viruses in RP populations. The first hypothesis suggests that there is an independent RP cell type that is chronically infected by OtV5 and slowly releases viruses via budding. The second hypothesis assumes that RP populations contain mainly resistant cells with a small proportion of susceptible cells that maintains the viral population through infection and lysis. I developed two epidemiological models to test these two hypotheses and explored how well each model explains the experimental data available. Although neither model reflected the experimental data perfectly, I show that a mixed population of susceptible and resistant cells can explain the population dynamics seen in RP populations. My results demonstrate the utility of using epidemiological models to examine host-virus population dynamics in marine systems.

5.2 Introduction

Hosts employ multiple strategies to confer full or partial resistance against a pathogen. The type of resistance strategy used by the host will have both ecological and epidemiological consequences. Bacteria-phage epidemiological dynamics have been studied extensively due to the relative ease of using these study systems in the laboratory and it is widely accepted that phage can drive evolution in their bacterial hosts on the genome, population and community levels (Koskella and Brockhurst, 2014). In contrast to bacteria, we know less about resistance and epidemiological dynamics in eukaryotic single-celled marine algae and their viruses. This is largely for three reasons: (1) the vastness and three-dimensional structure of the marine

environment, making it difficult to sample; (2) the difficulty of co-culturing marine algae and viruses in the laboratory; (3) the movement restriction that small culture flasks impose on the system, preventing host movement into or out of that environment, meaning that virus infection usually leads to a population crash.

Ostreococcus tauri is a picoeukaryotic green alga that has been widely adopted over the past decade to study marine virus infection. *Ostreococcus tauri* viruses (OtVs) are abundant in Mediterranean lagoons, where their host is also abundant, and are mainly species specific (Clerissi *et al.*, 2012). *O. tauri* can be susceptible (S) to virus infection, upon which infected cells are lysed. However, resistant (R) cells always arise in laboratory cultures as a small proportion of the population, which is expected to be at least one in 1000 (Yau *et al.*, 2016). When these few resistant cells divide to found a population, that population is made up of resistant cells; these cells remain resistant upon re-infection, demonstrating that the resistance is heritable and stable. Some resistant populations of *O. tauri* have been found to coexist with infectious viruses in laboratory cultures (Thomas *et al.*, 2011; Yau *et al.*, 2016), providing potential for a coevolutionary relationship to exist between the host and virus. These populations are referred to as resistant producers (RP) because new viruses are being "produced" within the resistant populations.

When RP populations were first isolated, the available evidence pointed towards RP cells having an independent resistance type, where cells were chronically infected and released viruses slowly via budding instead of lysis (Thomas *et al.*, 2011). However, this view was recently revised in the light of new data showing that a small proportion of cells were undergoing lysis. Based on this new data, the proposed revised mechanism is that the viruses in the RP populations are being produced by the lysis of susceptible cells that exist as a small proportion of a mainly resistant population (Yau *et al.*, 2016). This mechanism is consistent with the observation that eventually infectious viruses are no longer detected in RP populations, although the time it takes for virus production to stop is variable, from a few weeks (Heath *et al.*, 2017, Chapters 3 and 4) to more than two years (Yau *et al.*, 2016). After virus

production has stopped, RP populations remain resistant to lysis upon exposure to new virus infection.

In this chapter, I developed two mathematical epidemiological models to investigate how the population dynamics of RP *O. tauri* populations change over time, with the aim of shedding light on how virus populations are maintained. The first model assumes that resistant producers (RP) are a distinct cell type, where infected cells do not lyse but instead shed viruses by budding (Thomas *et al.*, 2011). The second model tests whether the RP phenomenon can be explained by a mixed population consisting of both S and R resistance types (Yau *et al.*, 2016). If the first model reflects the mechanism for RP type, the RP resistance type could be a costly defence strategy, because the cell must tolerate chronic virus infection while avoiding lysis, and therefore there would be a strong selection pressure for RP cells to be lost. Since viruses are present in the population, resistance should be selected for, which would explain why we see RP populations maintaining their resistance to lysis and becoming R, rather than S. On the other hand, if the second model reflects the mechanism, and RP populations consist of a mixed S and R population, S cells must grow at a frequency sufficient to propagate viruses over subsequent generations. As above, the presence of viruses in these cultures imposes a strong selection pressure for resistance and we expect the proportion of S to decrease over time, as R is selected for. A shift from RP to R has been shown (Yau *et al.*, 2016; Heath *et al.*, 2017), therefore we would expect S to reach zero in order for the virus population to go extinct, and R to reach N (total maximum population size). For this chapter, I also performed laboratory experiments to measure OtV5 decay rate, the rate at which the virus OtV5 stops being infectious, as this model parameter was previously unknown.

5.3 Experimental procedures to measure OtV5 decay rate

In order to provide an estimation of the decay rate of OtV5 that could be embedded in the models as a parameter, I first performed laboratory experiments. Here, virus decay refers to loss of infectivity, since infectivity could be lost before the physical destruction of the virion particle (Fuhrman, 1999). A suspension of OtV5 was split

into five replicates of 10 ml in 15 ml Falcon tubes. The suspensions were incubated at 18°C in a 14:10 hour light:dark cycle, which is identical to the incubation conditions of the *O. tauri* host. To measure the number of viruses in the suspensions, a liquid lysis technique was used. A series of ten-fold dilutions was made for each replicate using 96 wells plates and 10 μl of each dilution was added to 1 ml of exponentially growing susceptible *O. tauri* strain RCC4221. Negative controls containing no OtV5 suspension were performed. Plates were sealed with Parafilm and incubated at 18°C in a 14:10 hour light:dark cycle. Wells were checked for clearing up to a maximum of 10 days. This test was performed every 7 days.

The number of cleared wells, indicating cell lysis had occurred, is shown in Figure 5.1. The method used for estimating virus abundance meant that there was a higher resolution for lower numbers of infectious viruses than for higher numbers (Figure 5.2). This was an artefact of the experimental design; the range of viruses increased with each lysed well due to the increasing dilution factor. Over a period of 69 days, there was a mean loss of 1.45% of the infectious OtV5 population per day. This is lower than the estimated decay rate for *Emiliania huxleyi* viruses of 0.1 to 0.8 per day (Bratbak *et al.*, 1993).
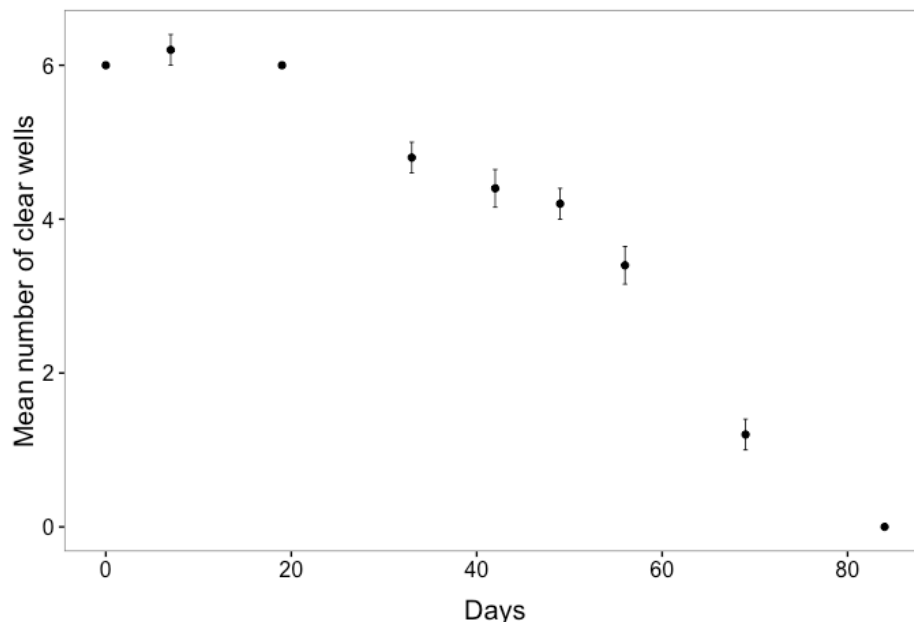
Figure 5.1. OtV5 decay rate as measured by the number of clear wells following 10-fold serially-diluted OtV5 inoculation. Data points are mean ± standard error (n=5).
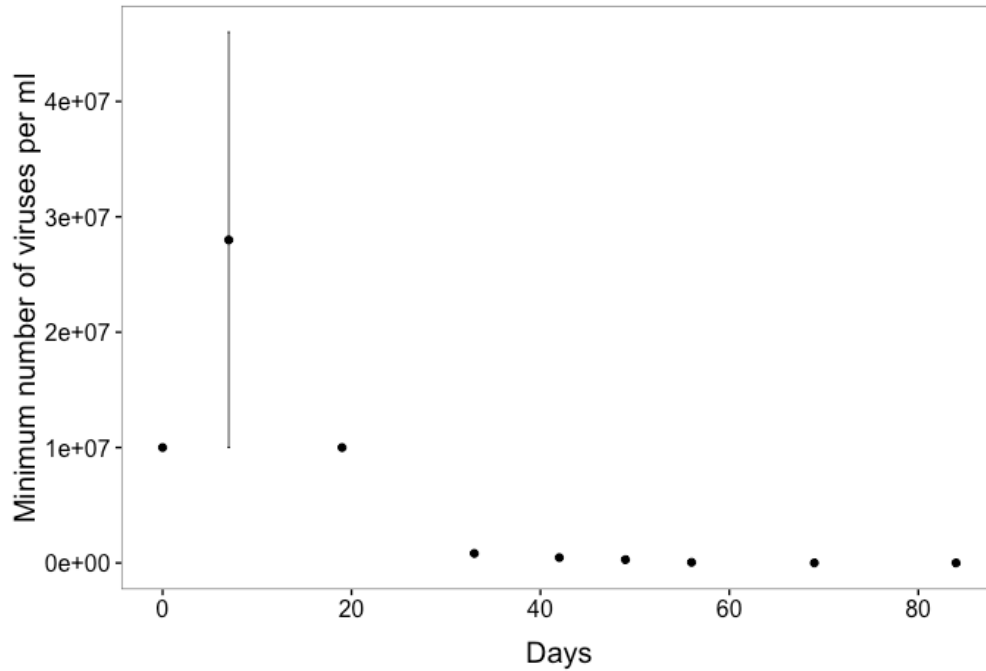
Figure 5.2. Estimated minimum number of infectious OtV5 particles per ml. Mean ± standard error (n=5).

5.4 Mathematical modelling

I used modified SIR (Susceptible-Infected-Resistant) type mathematical models (Kermack and McKendrick, 1927) to explore the RP resistance type at the level of a population by comparing the likelihood of two models being correct given my experimental data from Chapter 4. This type of framework is commonly used for epidemiological models of disease spread and similar models have also been used to study virus infection in bacteria (Lenski, 1988; Middelboe, 2000). I performed all model simulations and approximations of ordinary equations using the Runge-Kutta methods using the deSolve package in R (version 3.3.1). Parameter values for the two models are defined in Table 5.1.

Table 5.1. Parameter values for the RP and SIRV models.

Parameter	Description	Value	Reference
b	Birth	1 division per day	Heath & Collins 2016
d	Death	0.05	Estimate
β	Adsorption rate	20-35%	Derelle *et al.* 2008
λ	Lysis rate	0.99	Estimated based on Derelle *et al.* 2008
r	Conversion of RP to R	0.3	This study
p	Virus production rate from RP cells	3 viruses per cell per day	Thomas *et al.* 2011
α	Conversion rate of I to R	0.01%	Yau *et al.* 2016
γ	Virus decay rate	0.01	This study
k	Carrying capacity	1 000 000	This study

5.4.1 RP model – RP cells are a discrete resistant type

5.4.1.1 Description of the RP model

My first model, referred to as the RP model, examines a population consisting of a single RP resistance type (Figure 5.3). This model assumes that the culture is well mixed and that *O. tauri* cells and OtVs are distributed homogenously and come into contact at random. The starting population is comprised only of RP type cells. RP cells reproduce to have RP offspring at rate b and die at rate d. RP cells produce viruses and release them to the environment at a rate of 3 viruses per cell per day (p), as was measured from experimental observations (Thomas *et al.*, 2011). RP cells stop producing viruses at rate r, at which point they shift to become R type cells. Importantly, we know that R *O. tauri* populations persist over at least 200 generations in the absence of OtV (Heath *et al.*, 2017). Thus, resistance is inherited

with a high frequency, such that R offspring are also R, with no measurable reversion to the RP resistance type. I do not include the possibility of RP shifting to the S resistance type, since as of yet this has never been observed in the laboratory (Thomas *et al.*, 2011; Yau *et al.*, 2016; Heath *et al.*, 2017). Since resistance involves a chromosomal rearrangement in this system, it makes sense that reversion from R or RP to S host types is extremely unlikely. The virus (V) population is populated only from the production of viruses by RP cells, and the only losses are from decay (γ) and attachment to RP and R cells (β), which does not result in successful infection or lysis. Viral decay rate γ is the loss of infectious viruses from the population, both through loss of infectivity and physical decay of the particle, and is constant and proportional to virus population density. I assume that population growth for all three resistance types is density dependent and limited by the carrying capacity, *k*. The structure of the RP model is shown in Figure 5.3.
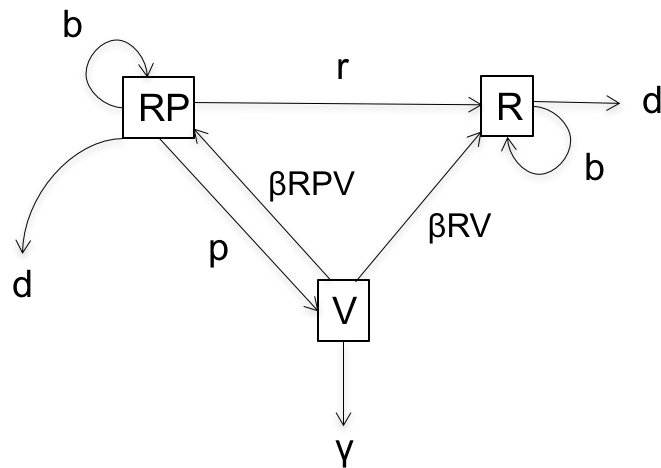
Figure 5.3. Flow diagram of the RP model. Boxes represent the populations of resistant producer cells (RP), resistant cells (R) and viruses (V). Arrows exiting and entering boxes represent when a process removes an amount of a variable or contributes to an amount of a variable, respectively.

The population dynamics for the RP model can be expressed in the following ordinary differential equations:

$$\frac{d\text{RP}}{dt} = b\text{RP}\left(1 - \frac{N}{k}\right) - d\text{RP} - r\text{RP}$$

$$\frac{d\text{R}}{dt} = b\text{R}\left(1 - \frac{N}{k}\right) - d\text{R} + r\text{RP}$$

$$\frac{d\text{V}}{dt} = p\text{RP} - \gamma\text{V} - \beta\text{RPV} - \beta\text{RV}$$

where total cell population size $N = RP + R$.

5.4.1.2 RP model outputs

The RP model predicted that the resistant producer RP and virus V populations would decrease and eventually go extinct (Figure 5.4). However, the V population crashed almost immediately, which is not representative of experimental data showing some cases where viruses can persist in RP populations for months or years (Yau *et al.*, 2016, Chapter 4).

The parameter values tested for the RP model are listed in Table 5.2. Higher viral production rate of RP cells (p) increased the initial spike in density, but did not affect the virus population dynamics overall. This is because although viruses are constantly being produced by RP cells, any viruses will attach to R and RP cells, subsequently being removed from the population. The high density of cells means that viruses come into contact with a cell almost immediately, so there are never free viruses. The low numbers of viruses being produced indicates that virus decay rate γ is negligible, since all viruses attach to a cell before they have a chance to decay.
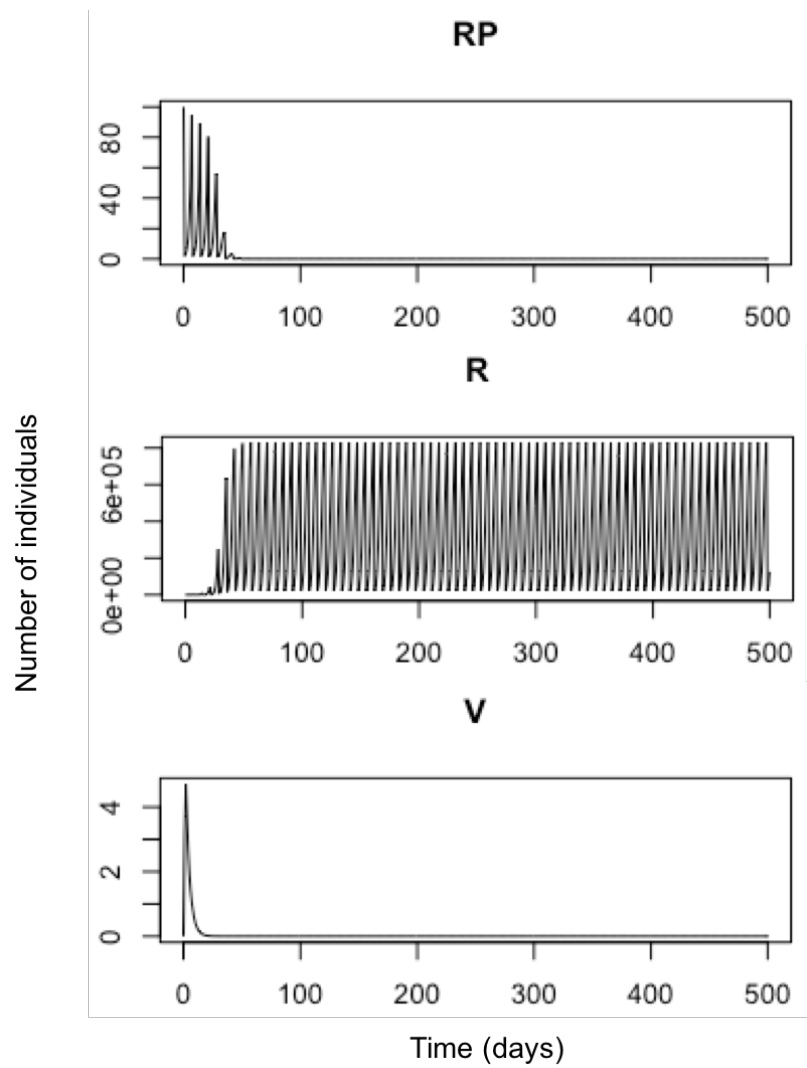
Figure 5.4. Predictions from the RP model. Parameters: $b = 1$, $d = 0.05$, $\beta = 0.35$, $\gamma = 0.01$, $r = 0.3$, $p = 3$, $k = 1,000,000$. Note that the model outputs for each population are on different scales, to facilitate visualisation of the population dynamics.

Table 5.2. Parameter values tested with the RP model

Parameter	Description	Values tested
b	Birth	1
d	Death	0.05
β	Adsorption rate	0, 0.35
r	Conversion of RP to R	0.29, 0.3, 0.31, 0.4
p	Virus production rate from RP cells	3, 6, 10, 25
γ	Virus decay rate	0.001
k	Carrying capacity	1 000 000
D	Dilution factor	0.01

To model viruses persisting in the population for several months, as previously observed in some RP populations, I relaxed the assumption that viruses adsorb to resistant cells, setting β to zero. When β was removed, the V population showed similar dynamics to the RP population (although the V population was approximately four times greater than the RP population) and gradually decreased before becoming extinct (Figure 5.5). Because the only shift in population dynamics is from RP to R, it is inevitable that this leads to RP (and therefore also V) extinction. Once the RP population has become extinct, there are no hosts available for virus replication.
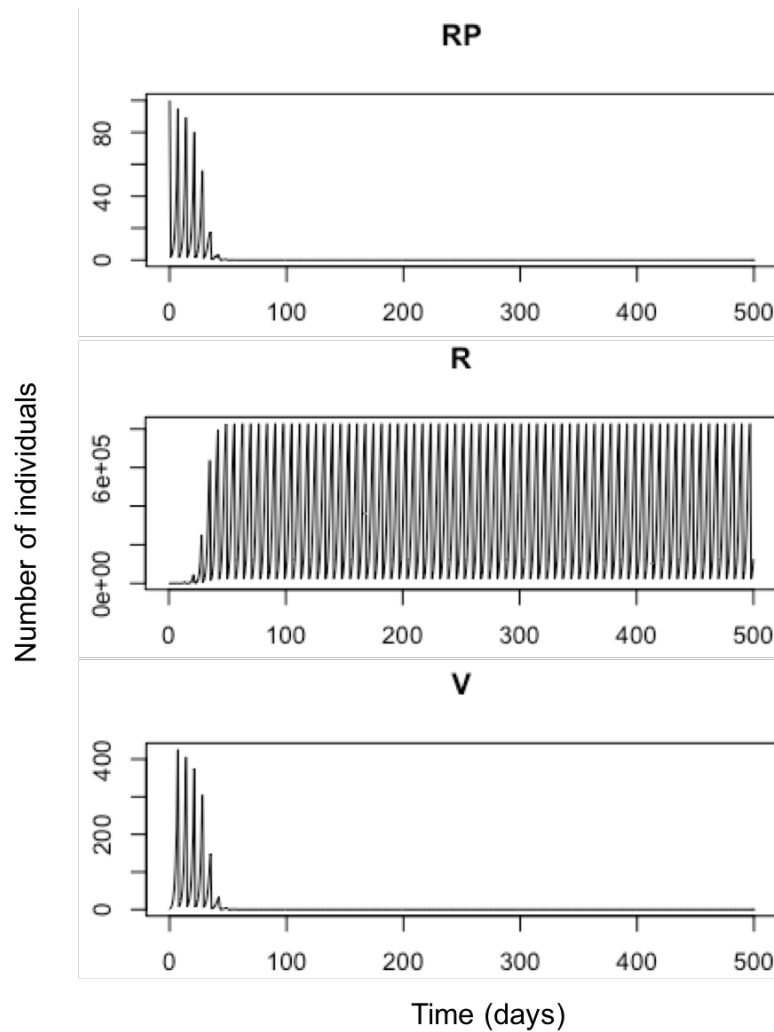
Figure 5.5. Predictions from the RP model when β is removed, thus preventing V being removed from the population via attachment to *O. tauri* cells. Parameters: *b* = 1, *d* = 0.05, *γ* = 0.01, *r* = 0.3, *p* = 3, *k* = 1,000,000. Note that the model outputs for each population are on different scales, to facilitate visualisation of the population dynamics.

The R cell population grew exponentially but was always diluted by serial transfer before it reached carrying capacity, *k*. The model predicts that the rate at which RP cells stop producing viruses and become R (*r*) is very important and must be low for the RP and V populations to be maintained. The higher the value of *r*, the faster the RP and V populations decrease.

5.4.2 SIRV model – RP populations consist of R and S cells

5.4.2.1 Description of the SIRV model

The second model assumes that an RP population consists of susceptible (S), infected (I) and resistant (R) host cells and viruses (V) (Figure 5.6). Similarly to the RP model, the SIRV model assumes the culture is well mixed and that host *O. tauri* cells and OtVs are distributed homogenously and come into contact at random. The rate at which an encounter between a host cell and an OtV results in a successful infection is the product of susceptible host cell density (S), OtV density (V) and the adsorption rate constant (β). The encounter rate is proportional to the density of the host and V. Successful infection of S causes cells to become infected (I) and usually results in cell death via lysis (λ) with a burst size of 25 viruses per lysed cell (Derelle *et al.*, 2008). However, some I cells can become resistant (R) at rate α. This is in line with previous observations that R cells arise spontaneously in *O. tauri* populations exposed to OtV (Thomas *et al.*, 2011). As above, resistance is inherited and R cells produce R offspring, which persist in the population. OtVs are also able to adsorb to R cells in the same way as they do to S cells (βRV), (Thomas *et al.*, 2011) but do not cause cell lysis in R cells, suggesting that the viral resistance mechanism in *O. tauri* is intracellular. This adsorption rate is important, since attachment of viruses to both S and R cells removes free viruses from the population. Viruses are also removed from the population via decay of the particles (γ), which is constant and proportional to the V population density. For simplicity, β, λ, burst size and γ are assumed to be constant. The carrying capacity for the population is defined as *k*.

The SIRV model assumes that the starting population already consists of S and R cells, with S present at a proportion of 1/1000, as this is what has been predicted previously (Yau *et al.*, 2016). Infectious OtV5 viruses (V) are also present in the starting population at a proportion of 50 times less than the cell population, which was the average density measured in Chapter 4.
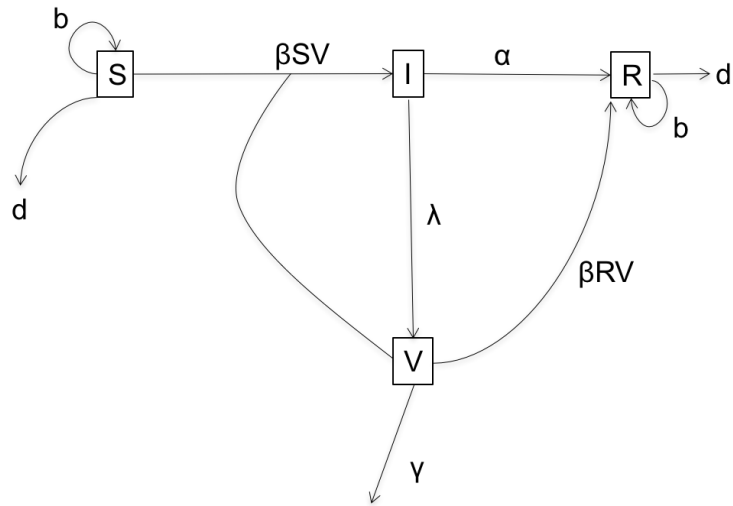
Figure 5.6. Flow diagram of the SIRV model. Boxes represent the number of susceptible (S), infected (I), and resistant (R) *O. tauri* cells and viruses (V). Arrows exiting and entering boxes indicate when a process removes an amount of a variable or contributes to an amount of a variable, respectively.

The population dynamics for the SIRV model can be expressed in the following ordinary differential equations:

$$\frac{dS}{dt} = bS\left(1 - \frac{N}{k}\right) - dS - \beta SV$$

$$\frac{dI}{dt} = \beta SV - \lambda I - \alpha I + bI\left(1 - \frac{N}{k}\right)$$

$$\frac{dR}{dt} = bR\left(1 - \frac{N}{k}\right) - dR + \alpha I$$

$$\frac{dV}{dt} = \lambda I \cdot 25 - \beta SV - \beta RV - \gamma V$$

where total cell population size $N = S + I + R$.

5.4.2.2 SIRV model outputs

The SIRV model was run to predict what would happen if three host cell types (S, I and R) and viruses (V) coexisted in culture. The parameter values tested for this model are given in Table 5.3. The densities of the S and R populations oscillated due to the dilution from serial transfer (Figure 5.7). The results from this model show that the S and R populations become relatively stable and coexist. S does not go extinct, in contrast to what is eventually expected from experimental observations. However, the V population does go extinct which also leads to the extinction of I since there are no longer any viruses available to infect cells. Regardless of how the parameter values were manipulated, no population went extinct. Additionally, the proportion of S cells within the total population size was always higher than has been observed in laboratory cultures (Yau *et al.*, 2016). Adding a cost of resistance to the model, no matter how small, always caused the R population to go extinct, and therefore was not included since it did not represent the experimental data. In this model, the lysis rate λ, was high, because all infected susceptible cells are expected to die, except those that become resistant. Thus, as λ was decreased, I increased, as expected. When λ was 0.55, approximately half of what would be expected, the infection patterns remained similar except I abundances were higher.

Table 5.3. Parameter values tested for the SIRV model

Parameter	Description	Value
b	Birth	1
d	Death	0.05
β	Adsorption rate	0.35
λ	Lysis rate	0.55, 0.99
α	Conversion rate of I to R	0.01, 0.04, 0.075, 0.35, 0.5
γ	Virus decay rate	0.0001, 0.0005, 0.001, 0.01, 0.8
k	Carrying capacity	1 000 000
D	Dilution factor	0.01
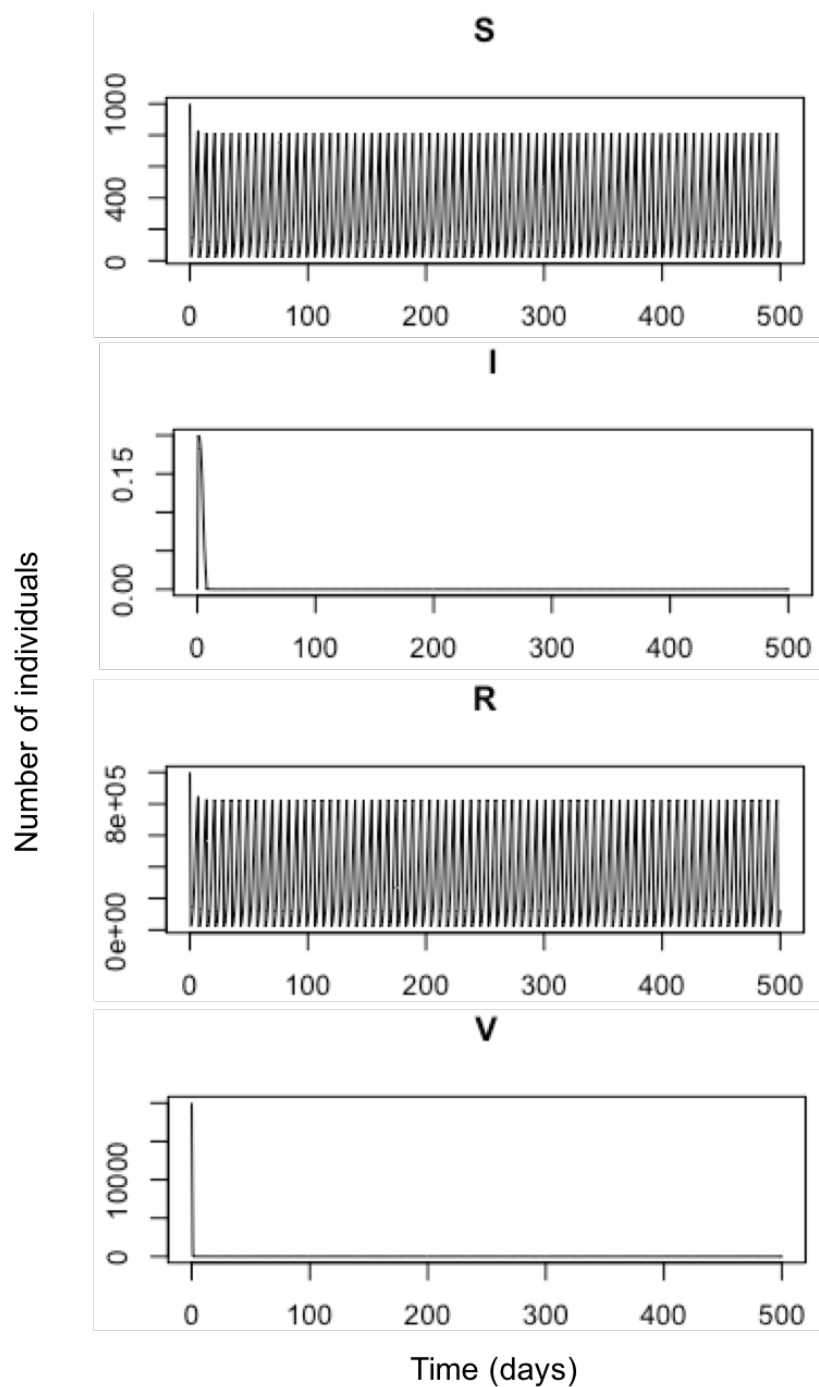
Figure 5.7. SIRV model output of the dynamics of susceptible (S), infected (I) and resistant (R) host *O. tauri* cells and OtV5 viruses (V). Parameters: $b = 1$, $d = 0.05$, $\beta = 0.35$, $\lambda = 0.99$, $\gamma = 0.01$, $\alpha = 0.01$, $k = 1\ 000\ 000$.

Virus decay rate γ was measured to be 0.015 per day from experimental data. I examined the effect of different decay rates by changing γ. Changing the value of γ did not affect the virus or host populations. Increasing burst size to 55 viruses per cell did not affect population dynamics, possibly because the number of infected cells was so low that the number of viruses could not increase by much even when burst size was large. However, large burst sizes for *O. tauri* are unrealistic because of the proportionally large size of the viruses compared to the small cell.

As with the RP model, the R population remains very stable with regular oscillations correlating with the serial transfers, and does not reach carrying capacity. R populations continue to grow until serial transfer because population growth of R is exponential, with some contribution from α (the mutation rate of I to become R); the removal of R cells only occurs through natural death d which is low due to the nature of the culturing conditions. Therefore, R populations do not reach k before serial transfer. I examined what would happen if the rate at which I become R (α) is increased in the model, because viruses are always present in RP populations meaning host resistance is under a constant selection pressure. When α was increased from 0.01 to 0.5, the I population decreased, but still reached extinction within the same time (8 days). With $\alpha<0.01$, there was little change in population dynamics.

In the SIRV model, the V population became extinct after only one day, in contrast to what I observed in my experiments in Chapter 4 (usually $1.2 \times 10^4 - 3.1 \times 10^5$ viruses per ml). In this model, I used a starting virus density of 50 times less than the starting density of *O. tauri* cells. In Chapter 4, I observed between 5 and 120 times as many cells as viruses in my cultures. When the starting density of V was increased in the model, I increased, however there was little change in S and no change in R.

5.5 Discussion

When susceptible (S) *O. tauri* populations are infected with OtVs, resistance is always observed. The proportion of cells inferring resistance has been estimated to be at least one in one thousand (Yau *et al.*, 2016). This could be because some cells already have a resistance mutation (Luria and Delbrück, 1943), however it is more likely to be a biological response that is induced upon exposure to viruses due to the chromosomal rearrangement observed (Yau *et al.*, 2016). The rapid chromatin restructuring in chromosome 19 suggests epigenetic modifications, which occur much more rapidly than genetic changes (Klironomos *et al.*, 2013). These changes are heritable, since resistant populations derived from a single clone continue to produce resistant offspring, even when they are grown without viruses (Yau *et al.*, 2016; Heath *et al.*, 2017). I modelled two scenarios for the RP phenomenon. Neither of these models was enough to fully explain experimental observations (Yau *et al.*, 2016; Heath *et al.*, 2017, Chapter 3). However, parts of both models explained some aspects of what has been observed. Additionally, I found that the SIRV model could partially explain the *O. tauri*/OtV5 infection dynamics, meaning there may be no need for a third resistance type.

5.5.1 RP model

The RP model predicted that resistant producer RP and virus V populations would go extinct and the final culture would consist of only resistant R cells. In this way, it supports what has been observed in experiments, as eventually no viruses are detected in cultures (Yau *et al.*, 2016; Heath *et al.*, 2017). However, in the RP model, the V population drops very quickly before becoming extinct. This is not what I found in my experiments (Chapter 4), which showed that the number of infectious virus particles could fluctuate, and did not always decrease.

The RP model showed that all V would adsorb to *O. tauri* cells as soon as they were released from RP cells, thus not allowing for any free viruses within the culture. In contrast, in experimental cultures, there is a high proportion of free virus particles (Chapter 4). This could be for two reasons. First, RP cells could be releasing more

than three viruses per day, which was the value used in the model and predicted by Thomas *et al.* (2011). This is unlikely, since RP cells need to produce viruses without lysis, which means the rate of production should be lower than a lytic infection. Even when the virus production *p* was increased to 25, the virus V population went extinct rapidly. Second, viruses are either not attaching to RP and R cells, or they are attaching at a lower rate than previously reported. Previous observations found that there was no significant difference in attachment of OtV5 to R and RP cells compared to attachment to S cells (Thomas *et al.*, 2011). This is not surprising if the resistance mechanism is intracellular and induced by exposure to viruses, as suggested by Yau *et al.* (2016). Thus, for attachment (*β*) to be lower, R and RP cells might evolve a different cell surface morphology preventing adsorption of viruses to the cell surface. If the resistance mechanism is intracellular, there is not a strong selection pressure for resistant cells to modify cell surface receptors to prevent virus attachment. I suggest a further possibility for lower attachment is that the viruses can adsorb but release rapidly, such that they are not always stuck to cells. This could explain why such a large number of viruses is detected in laboratory cultures. If this is the case, an improved model could capture this important aspect of host-virus interaction by including detachment of the viruses. In my model, I examined this by decreasing adsorption rate *β*.

5.5.2 SIRV model

The SIRV model did not predict the susceptible S or resistant R populations to go extinct, but the virus V population went extinct immediately. Running the model with different parameter settings did not result in a better fit between the model and the data. The dilution factor introduced by sub-culturing in the models was the same as that used in experiments in Chapter 4 and caused all populations to fall to low densities. The sub-culturing the S, I and V populations result in periods of low population size, and it is possible that at these times extinction could occur (Levin *et al.*, 1977). I suggest that extinction of a host resistance type or virus could occur when the population is low and unable to recover. For example, if there were a slight increase in burst size and all S cells became infected, this could lead to extinction of

S and I. On the other hand, the V population could become extinct if there were an increase in viral decay rate, a decrease in adsorption rate or a decrease in contact rate with S cells. Additionally, a higher adsorption rate could lead to more viruses attaching to R cells which would remove viruses from the population. If there is a density threshold below which populations have a higher probability of going extinct, this could explain why we see such large variation in the length of time it takes for infectious viruses to stop being detected across the RP populations. If the numbers of S cells and V regularly drop very low, very small changes in the parameter values could lead to cases in which the populations are prevented from increasing again. If this is so, the SIRV model can partially explain the infection dynamics of *O. tauri*/OtV5 and therefore means that a second type of resistance, i.e. the RP cell type, is not necessary to explain how these cultures maintain a virus population for up to hundreds of generations.

The SIRV model assumes that infected populations do grow. Infected *O. tauri* cells containing viruses have been seen dividing (Yau *et al.*, 2016), however these experiments only observed around 0.5% of infected cells within a population at any given time. Additionally, since I assume that all infections result in cell lysis, which occurs rapidly in approximately 8 hours (Derelle *et al.*, 2008), I assume that infected cells are not competing for resources with uninfected susceptible and resistant cells. Some cyanobacteria species remain able to photosynthesise following virus infection (Suttle and Chan, 1993). However, in a model of bacteriophage, Lenski and Levin (1985) assumed that infected cells neither use resources nor grow. In this model, cells are not growth limited and are kept in exponential growth, and allowing growth of I did not prevent growth of S or R.

Yau *et al*. reported 0.5% of their RP populations as undergoing viral infection (Yau *et al.*, 2016). In my SIRV model, the infected population quickly became extinct, because the extinction of the virus population meant that susceptible cells would no longer become infected. In the SIRV model, the S population was 750 times lower than the R population meaning that this aspect of the model could explain the RP phenomenon, since the proportion of S was small enough that there was no

detectable drop in N following OtV5 inoculation. Studies on OtV5 inoculation found no significant difference in RP population density following OtV5 inoculation (Heath and Collins, 2016; Heath *et al.*, 2017), meaning that for the SIRV hypothesis to hold true, S must not exceed 0.5% of the population.

5.5.3 Can the RP model or the SIRV model explain the RP phenomenon?

We know that unicellular marine algae can acquire resistance to virus infection, and Fuhrman (1999) suggested that in fact resistance is dominant and that viral production is not as high as it appears, although this is not consistent with the high virus production observed in the oceans (Wigington *et al.*, 2016). If this suggestion is correct, it could support evidence for the SIRV model, where there is a mainly resistant population.

One explanation for why neither the RP model nor the SIRV model accurately represented what we observe in experimental studies is that the parameter values are fixed. The experimental results from Chapter 4 showed a huge variation in the loss of virus production from RP cultures, therefore, one model is not sufficient to explain what is happening. It is possible that *in vitro* parameter values are changing, and small changes could have large consequences leading to the rate of extinction of viruses to vary from almost immediately, to several months. For example, the RP model showed that *r*, the rate at which RP cells stop producing viruses, is an important factor for the time it takes for RP and V populations to go extinct. If the RP model holds true, variations in *r* could explain how we see such huge variations in the loss of virus productions between cultures. However, it is unclear how *r* could change between lines and replicates by such a great amount. If the RP resistance type is costly, then we expect *r* to increase, because there is a selection pressure to stop producing viruses but to retain resistance to lysis while viruses are still present in the population. Experiments from this thesis (Chapter 4) and studies by others (Yau *et al.*, 2016) have found that there is a huge variation in the length of time that RP populations continue to produce viruses. This highlights the complexity of this

phenomenon and the difficulty in predicting the evolutionary and ecological consequences of this situation.

The shift from an RP population to R is expected regardless of the method of virus production, due to the selection pressure for resistance in the presence of viruses. However, we observe that following the loss of virus production in RP populations, populations remain resistant to lysis and there is no reversion to S, even after many generations in the absence of viruses where resistance is not required. This suggests that resistance is genetic and reversion to susceptibility is difficult or rare. I previously observed that resistance to OtV5 is not costly as measured by infectivity, growth and competition (Heath *et al.*, 2017, Chapter 3). Although evolutionary theory suggests resistance must be costly for susceptibility to persist (Bohannan *et al.*, 2002), other laboratory studies with bacteria have found that low or no cost of resistance is possible (Lenski, 1988).

5.5.4 Ecological relevance

The RP and SIRV models developed in this chapter are useful to start understanding the dynamics of the *O. tauri*/OtV system, however in some instances they may not be representative of the ecological consequences of virus production in the oceans. Both models predict extremely low virus abundance, which is not representative of the high levels of viral production in my experiments (Chapter 4) but is at the lower end of the range predicted in the ocean (Wigington *et al.*, 2016). The RP and SIRV models predicted maximums of 5 and 8 viruses per ml, respectively. Viral abundance estimates in the Leucate lagoon, where *O. tauri* was isolated, were between 5 and 20 000 viruses per ml (Bellec *et al.*, 2010). It is also difficult to extrapolate computer and laboratory studies to natural environments. For example, studies of coevolution with bacteria and phage have found that rapid mutation rates evolved within the laboratory (Pal *et al.*, 2007) but not in soil (Gómez and Buckling, 2013). One explanation for different results being observed in the field could be that the dynamics of natural microbial/virus systems are due to trade-offs and strain diversity, such that models of single strains of hosts and viruses are unlikely to capture all of

the dynamics of the system. We know that many strains of *Ostreococcus* viruses exist. For example, Clerissi *et al*. isolated 40 *O. tauri* viruses, all but two of which came from Mediterranean lagoons (Clerissi *et al.*, 2012). Thus we know that OtVs are much more abundant in lagoons and rare in open ocean sites (Bellec *et al.*, 2010; Clerissi *et al.*, 2012), probably because this is where the host is more often found, but possibly due to higher dispersal rates in the open ocean. Future experimental and computational work should consider study systems with multiple viruses and hosts to include a competitive element. This would tell us whether being resistant to one virus strain is relatively easy compared to being resistant to multiple strains.

5.6 Conclusions

In this chapter I have proposed two mathematical models to investigate further the infection dynamics of *O. tauri* with particular focus on the resistant producer phenomenon. I have shown that a combination of S and R cells is enough to explain the existence of RP populations. I conclude that since there is such huge variation in loss of viral production in RP cultures, a single model with static parameters is not sufficient to explain what is happening. Though neither model reflects the current available data perfectly, the SIRV model provides the most parsimonious interpretation. Thus, I suggest that these cultures are complex and there are one or more factors that must have high variability between cultures which leads to the large variability in viral loss.

6. Discussion

6.1 Purpose and significance of the study

The overarching goal of this thesis was to understand the evolutionary consequences of maintaining viral resistance using *O. tauri* as a study system. In this Discussion, I consider the implications that my results have on the evolutionary response of *O. tauri* to virus exposure and environmental change.

I have shown that host *O. tauri* cells that are resistant to virus infection can have a growth advantage across a range of environments (Chapter 2), that resistance to a single virus strain is maintained in the absence of viruses and does not have a detectable growth or competitive cost (Chapter 3), that virus abundance fluctuates in resistant producer lines and can be affected by phosphate concentration (Chapter 4), and that resistant producer lines have complex population dynamics and most likely consist of a mixture of susceptible and resistant cells (Chapter 5). My findings should be of interest to evolutionary biologists studying trade-offs and costs of resistance, as they show that even though a cost of resistance is predicted in theory, it can often be difficult to measure experimentally. My findings should also be useful for ecologists studying the responses of marine microorganisms to a changing environment, since I show that susceptible and resistant hosts can respond to abiotic environmental change in similar ways.

In this chapter, I will discuss the wider implications of my results and future directions directly arising from them.

6.2 Why is a cost of resistance so hard to find?

Understanding how and why a cost of resistance evolves is currently one important area of evolutionary biology, largely motivated by the increasing resistance of bacteria to antibiotics (Melnyk *et al.*, 2015) and resistance of crop pathogens to

pesticides (Bass *et al.*, 2015). In Chapter 3, I showed that there was not a cost of resistance in *O. tauri* in the fitness-related traits that I measured, and in Chapter 2 I showed that, in contrast to the expectation that there be a cost of resistance, resistant producers could have a growth advantage under some conditions. I offer four possible explanations for this which I will discuss: (1) there is not a cost of resistance in the laboratory; (2) a cost of resistance could be lost or reduced; (3) cost of resistance is too small to detect; and (4) there is a cost of resistance in *O. tauri* in a trait that I did not measure. Although this is not an exhaustive list, I have demonstrated that a cost of resistance in *O. tauri* does not affect growth rate or adaptation to any of the environments I tested.

6.2.1 A cost of resistance is not detected under laboratory conditions

Experimental evolution is a tool that enables us to use highly controlled environments and simplify the real world to make it easier for us to understand. However, this can also make it difficult to understand how these same processes play out in wild populations when one observes a negative result in the laboratory. For example, marine phytoplankton such as *Ostreococcus* often persist in near-starvation conditions; but these conditions are not useful for performing evolution experiments, where a reasonably high cell division rate is necessary to finish an experiment during a single PhD. A rich environment could explain why cost of resistance has been difficult to detect in the laboratory, both in *O. tauri* and other organisms, because some resource allocation trade-offs may only exist under nutrient limitation (e.g. Lenski, 1988; Lennon *et al.*, 2007). In addition to differences in the total nutrient levels between field and laboratory environments, phytoplankton experience gradual, sudden and fluctuating changes in lagoons and the open ocean, and may experience periods of limitation (Clerissi *et al.*, 2014), whereas I (and most other evolution experiments that do not explicitly study the effect of environmental fluctuations) use stable environments. Fitness trade-offs in some species may only appear under stressful conditions, for instance when the cell must re-allocate resources from virus resistance to different survival mechanisms, such as nutrient receptors (Menge and Weitz, 2009). Studies into the epistatic interactions of resistance in bacteria found

that cost of resistance was highly dependent on the environment in which the fitness measurements were assayed in (Bohannan *et al.*, 1999; Meaden *et al.*, 2015). Additionally, a study using *E. huxleyi* found an effect of the laboratory culturing conditions, with strains that had been cultured in vitro for longer having lower viral production capacity (Ruiz *et al.*, 2017). Mesocosms could be used to investigate costs of virus resistance within more ecologically realistic environments, since they include natural environmental variables such as the presence of other species and levels and fluctuations of physical variables (e.g. light, temperature, salinity).

6.2.2 Selection and compensatory mutations can reduce or eliminate the cost of resistance

Cost of resistance can be reduced through selection against individuals with high costs or through compensatory mutations. In cases where there is a cost of resistance, there will also be strong selection within a population for genotypes/lineages with the lowest costs of resistance, and novel mutations that can lower that cost even more will rapidly fix in the population, causing the population to evolve an increasingly lower cost of resistance (Lenski, 1988a). Interestingly, when a cost of resistance is observed, it is often only present in a proportion of the population or strains tested (Lennon *et al.*, 2007; Avrani *et al.*, 2011). This points towards compensatory mutations being common and also responsible for a lack of cost of resistance, since some individuals display signs of a cost while others do not. Compensatory mutations could ameliorate the growth rate cost (Avrani and Lindell, 2015) with rapidly growing resistant cells out-competing slow growing resistant cells. This was demonstrated in *E.coli*, where compensatory mutations reduced the cost of resistance by half after 400 generations of evolution in the absence of phage (Lenski, 1988b). Laboratory selection experiments have high mutational supplies and select for fast generation times (due to the nature of transferring the cultures) (Bell, 2008), meaning that if compensatory mutations are possible they are likely to occur and increase in frequency in populations. In contrast, natural populations may have much slower growth rates, or may experience long periods of time without dividing (for example, if they are nutrient- or light-starved). Additionally, many traits that are under

selection in the ocean, such as those that are associated with avoiding grazers, would be neutral in my experiments. Thus, pleiotropic effects associated with compensatory mutations in natural populations may be less common in the laboratory, which would increase the prevalence of compensatory mutations by increasing the range of mutations that could compensate a cost of resistance in the laboratory. The suggestions outlined above could lead to higher levels of resistance in the laboratory than in natural populations, although experiments with natural populations would need to be performed to confirm this.

In Chapter 2, I showed that two out of three RP lines of *O. tauri* can have a growth advantage in some environments. This shows that there can sometimes be a growth benefit to being resistant to viral lysis. Furthermore, I found that population growth rates of resistant and susceptible cells, including the fast-growing RP lines, were equally low under low phosphate conditions, suggesting that there is not a cost (or benefit) of resistance under nutrient limitation in *O. tauri*. Following these results, in Chapter 3, I showed that after evolution in different environments, a cost of resistance was still not detected, and fast-growing RP lines had decreased their growth rates to that of the other lines. This provides new information on resistance in marine eukaryotic algae. The evolution of compensatory mutations could offer an explanation for these results.

Evolutionary theory assumes a cost of resistance in order for susceptible cells to persist. Only long-term studies over many generations would be able to detect subtle differences associated with costs. Is it possible that there does not have to be a cost of resistance? If resistant and susceptible cells occupy different niches (e.g. spatial or ecological), this may not necessarily lead to one phenotype being fitter. Alternatively, if the system is dominated by Kill the Winner dynamics, which is expected in many marine systems, being common would be a cost in itself (Thingstad, 2000). This is because "winning" strains with high densities will be more susceptible to predation from viruses (and grazers). There was no frequency-dependent selection in my experiments, meaning any costs that are frequency-dependent would not be detected. My experiments only allowed me to detect if there

were growth costs. I did not find any reversion of resistant lines to susceptibility, and studies using *E. coli* have found similar results, with hosts evolved in the absence of viruses maintaining and even gaining viral resistance (Lenski, 1988a; Meyer *et al.*, 2010). These experiments show that selection for resistance is complex and simple trade-off models are not able to predict the evolution of host resistance (Meyer 2010).

6.2.3 Cost of resistance is too small to detect

It is possible that some laboratory methods are not sensitive enough to detect a cost of resistance (Lennon *et al.*, 2007). Even if trade-offs are too small for us to observe, they could be large enough to have ecological impacts, for example by allowing many species or strains to coexist (Bohannan *et al.*, 2002). The smallest decrease in competitive ability could have large ecological consequences in the open ocean, where organisms must face many environmental challenges such as resource limitation or predator avoidance, thereby allowing viruses and algal hosts to stably coexist. The magnitude of a trade-off is important in determining whether resistance will persist (Bohannan *et al.*, 2002). However, the type of trade-off is also likely to be important. For instance, in *Prochlorococcus*, the growth cost of resistance declined over time, but enhanced infection was only lost in one out of five substrains (Avrani and Lindell, 2015). If a stronger selection pressure is exerted on growth rate due to competition, this could explain why we rarely observe growth trade-offs in culture, whereas selection to reduce costs (such as enhanced infection or reduced competitive ability with other species) will be dependent on space and time.

Population growth rate can be useful to measure adaptation of single celled algae to a new environment (Schaum *et al.*, 2012; Schaum and Collins, 2014; Brennan and Collins, 2015). However, I found that growth rate was not suitable to measure cost of resistance in this system, either because my measurements were not sensitive enough to detect subtle changes or because reduced growth rate is not a fitness trade-off for *O. tauri*. It is possible that since *O. tauri* does not usually form dense blooms (O'Kelly *et al.*, 2003), selection on this trait might be relatively weak relative to

other traits, such as those associated with nutrient acquisition. This is in line with its size, as most low-nutrient phytoplankton specialists in the ocean are small (Raven, 1998; Finkel *et al.*, 2010; Peter and Sommer, 2015), and it suggests that a cost of resistance could lie in a different trait from one of those that I measured.

6.2.4 Is there a cost of resistance in O. tauri that has not been identified?

A trade-off could be manifested as a trait that has not been measured in the laboratory. I suggest that being resistant to a single viral strain, in this case OtV5, may pose little or no cost, but that the fitness cost increases as hosts must become resistant to more viral strains, as is necessary in natural environments where *O. tauri* is exposed to hundreds of OtVs (Bellec, Grimsley, and Desdevises, 2010; Clerissi *et al.*, 2012). In other words, I speculate that there is a fitness trade-off with the number of viral strains a host can maintain resistance to. For all of my experiments, I only used a single strain of *O. tauri* and a single strain of OtV. Future work can build on this and add complexity by performing cross-infectivity tests using multiple strains of the host and virus. If this suggestion is correct, a cost could either be manifested as a limit in the number of virus strains against which a host is resistant, or as reduced fitness (e.g. growth rate or competitive ability) with increased number of virus strains to which a host is resistant. The combined cost of resistance in *E. coli* to two bacteriophage was less than expected compared to the separate fitness costs of being resistant to each bacteriophage (Bohannan *et al.*, 1999), but this study was still using only two viruses and cost could increase with number of phage against which the host is resistant. Alternatively, a trade-off of being resistant in *O. tauri* could be enhanced infection, as observed in cyanobacteria (Avrani *et al.*, 2011; Marston *et al.*, 2012), where increased resistance to one viral strain means increased susceptibility to other strains. In these cases, we might expect populations in the open ocean to have different magnitudes of resistance (Avrani and Lindell, 2015).

Resistance in *O. tauri* is stable and no reversion to susceptibility has been observed (Thomas *et al.*, 2011; Heath and Collins, 2016; Yau *et al.*, 2016; Heath *et al.*, 2017). This strongly suggests that there is a genetic constraint preventing susceptible cells

evolving from resistant ones, since cells remain resistant even when there is no selection pressure for them to be. Resistance is positively correlated with the size of the hypervariable chromosome 19, and it has been suggested that there could be a metabolic cost of resistance associated with production of a larger chromosome (Blanc-Mathieu *et al.*, 2017). However, this has not been observed through lower growth rates or cell size, and was only observed in the plastic response of resistant lines evolved in the control environment having lower chlorophyll content than susceptible cells (Chapter 3). Different metabolic processes such as mitochondrial membrane potential could be measured to investigate this idea further. Additionally, there is strong evidence for sexual reproduction in *O. tauri* (Grimsley *et al.*, 2010; Blanc-Mathieu *et al.*, 2017). Recombination rate is negatively correlated with chromosome size (Blanc-Mathieu *et al.*, 2017), suggesting there could be a cost to meiotic reproduction in resistant cells. Transcriptomics could be used to identify genes that are differentially expressed in algal populations with different resistance types or grown in different environments.

All work performed on the viral resistance mechanism in *O. tauri* has used OtV5 (Yau *et al.*, 2016). I propose that resistance of *O. tauri* to other viral strains must be studied and compared to examine whether the chromosomal restructuring is different for resistance against different OtV strains. If chromosome 19 is rearranged differently in response to different strains of OtVs, this could offer an explanation as to why not all cells collected in natural seawater samples are resistant to OtV5 inoculation, as expected if the mechanism is genetic and irreversible. If chromosome 19 is required to be rearranged differently in response to different strains of OtVs, there could be a limit to the number of virus strains against which a host can be resistant.

6.3 Does cost of resistance affect evolutionary potential?

Although I was unable to demonstrate a cost of resistance in *O. tauri* lines exposed to environmental change, there could be differences in other species. Future research could examine environmental effects on a species that is known to show a cost of

resistance under normal laboratory conditions, such as some cyanobacteria (Lennon *et al.*, 2007; Avrani *et al.*, 2011; Marston *et al.*, 2012; Avrani and Lindell, 2015). This would address the question of whether evolving resistance to viruses affects evolutionary potential because of costs, or primarily through some other mechanism.

In Chapters 2 and 3, I found that environment did not affect resistance in *O. tauri* in the absence of viruses. This finding provides support for *O. tauri* resistance being a genetic mechanism that is difficult to reverse. It also contradicts evolutionary theory that there should be a cost of resistance in the absence of viruses (Bohannan *et al.*, 2002), since if resistance were a costly trait to maintain, we would expect it to be lost when the selection pressure for it is removed. However, if there is a strong genetic constraint to losing resistance (Yau *et al.*, 2016), there should also be strong selection to minimise the cost of resistance. There are two possible ways to explain why susceptible cells are found in natural populations. First, resistance is in fact reversible, but not under the laboratory conditions, time scale and population sizes that I used. Second, not all susceptible cells are killed by viruses in the oceans and therefore they increase in frequency when no viruses that can infect them are present. More work needs to be done to understand why we still find susceptible cells in seawater samples: if there really is no cost of resistance, we would expect all cells to be resistant when coexisting with OtV viruses, which are known to be abundant (Bellec *et al.*, 2010; Clerissi *et al.*, 2014). This is not possible because it would drive the virus population to extinction. As stated in section 6.2.4, this suggests that it is more likely that there is a cost of resistance in the ocean that I have not measured in the laboratory, such as increased susceptibility to other viral strains (Avrani *et al.*, 2011; Marston *et al.*, 2012). It is possible that the cost of resistance can in fact be large and ecologically important outside the range of environments that I tested.

6.4 Understanding population dynamics in RP lines

6.4.1 Using epidemiological models to study marine host-virus systems

Viruses affect the population dynamics of their hosts as well as host evolutionary trajectories. It remains unclear how viruses are produced in resistant producer (RP) *O. tauri* populations, although evidence from my experiments (Chapters 4 and 5) supports the prediction by Yau *et al*. (2016) that the populations consist of a mixed susceptible (S) and resistant (R) population. How can we fully understand what is happening in RP lines? Empirical studies can give us some insight into the processes that are happening but they cannot always explain why. Computational modelling offers a tool to simulate population dynamics and predict what might happen when different parameter values are manipulated to represent different ecological scenarios.

In Chapter 5, I asked whether it is possible to predict population dynamics of marine organisms using an epidemiological model. I applied a modified SIR model to the marine environment to predict *O. tauri* population dynamics. Many mathematical studies of microbial host-virus populations in the oceans extend previously established models, for example by adding a viral component to typical NPZ (nutrient-phytoplankton-zooplankton) food web models (Weitz *et al.*, 2015) or by manipulating parameters in the Kill the Winner model (Thingstad, 2000; Knowles *et al.*, 2016). By using an SIRV model, I was able to include the infected (I) proportion of the population in the model, which is important in showing how cells have one of two fates following virus infection: lysis or resistance. I know of one other case where a modified SIR model has been used to study marine viruses (Middelboe, 2000), in this case bacteriophage. By applying epidemiological models, which have previously been used to study human diseases (Phillips, 1997; Blower *et al.*, 2000) and bacteria-phage dynamics (Lenski, 1988), I have taken a novel approach to examine marine virus and host population dynamics.

6.4.2 Do RP lines provide a mechanism to explain the existence of susceptible cells?

The RP *O. tauri* lines could provide an explanation for why we find susceptible (S) cells in natural seawater samples. Data from Chapters 4 and 5 provided supporting evidence that RP lines consist of both S and R cells to maintain the virus population (Yau *et al.*, 2016). However, for the RP lines to give a mechanism for susceptible persistence, there must be a cost of resistance.

Lenski and Levin (1985) observed coevolving *E. coli* and phage populations in the laboratory. They interpreted their result to suggest that a minority sensitive host population supported the phage population, and that for sensitive and resistant hosts and phage to coexist, sensitive hosts must have a higher fitness than resistant hosts. In a similar manner to my SIRV model outputs in Chapter 5, the *E. coli* and phage population densities both oscillated over time. In the case of *E. coli*, natural isolates have been found to be resistant to phage, whereas laboratory strains have reverted to sensitivity, implying a competitive trade-off (Lenski and Levin, 1985). In Chapters 3 and 4, nearly all of my RP populations stopped producing infectious viruses and became resistant (R) populations. This supports findings by Yau *et al.* (2016), suggesting it is common in *O. tauri*.

In my laboratory RP cultures, there is no advantage of being susceptible, because cells cannot escape contact with viruses. However, I suggest that in the ocean, many subpopulations of *O. tauri* coexist, consisting of susceptible or resistant individuals. Susceptible cells will be able to persist, either if there is a cost of being resistant in nature, or if a high enough proportion of individuals is able to avoid infection by dilution through ocean current movement. Laboratory populations are restricted by the volume of the flask in which they are cultured, which does not allow movement into or out of the population. I suggest that the high levels of susceptibility seen in laboratory strains could be an effect of this artificial environment, which does not allow cells to escape infection that otherwise might have. The coexistence of susceptible and resistant hosts with infectious viruses provides a unique system to

study coevolution in the laboratory because these populations could be more representative of natural populations.

6.5 Potential consequences of using non-axenic cultures

Often, laboratory experiments are performed with axenic cultures, meaning they contain only a single species and are entirely free from all other contaminating organisms (Andersen, 2005). However, observations have shown that many algae grow more rapidly in the presence of bacteria, and evidence suggests this is because of an important symbiosis in which algae utilize B vitamins produced by the bacteria through direct interactions, allowing them to acquire these essential vitamins much more rapidly than by diffusion from seawater in which they are present in very low concentrations (Croft *et al.*, 2005, 2006). Bacteria have been detected in *O. tauri* cultures in the laboratory, even following treatment with antibiotics and are speculated to be important for maintaining healthy *O. tauri* populations (Abby *et al.*, 2014). I did not use axenic cultures for my experiments, and the presence of non-photosynthetic bacteria was suggested from flow cytometry analysis which showed many particles within the same size range as *O. tauri* that did not contain chlorophyll (although these possibly also represented dust and salt particles) (Appendix figure 6). This could have had important implications for the interpretation of the results. For example, all other species that were not *O. tauri* would have also been competing for nutrients. Since I used very nutrient-rich media, this probably did not have a large effect, but it is an important point to be aware of when interpreting experimental results. On the other hand, using non-axenic cultures was more representative of a natural algal population in which many species within a community are constantly interacting. The phycosphere, the region immediately surrounding a phytoplankton cell, is attracting increasing interest and now the importance of symbiotic relationships and direct cell-to-cell interactions in marine microorganisms is widely recognised (Seymour *et al.*, 2017).

6.6 Implications of only considering infectious viruses

In Chapters 4 and 5, I focused only on infectious viruses within the RP *O. tauri* cultures. There is a strong possibility that there were non-infectious OtVs present within the cultures which I would not have detected using the liquid lysis technique. One important consequence of non-infectious viruses being present within the RP cultures is that they could have competed with infectious viruses, for example if they were still able to attach to the cell: attachment of non-infectious viruses would limit attachment of infectious viruses. As discussed in Chapter 4, presence of total OtV (infectious and non-infectious) can be detected using PCR. However, this requires a reasonably large quantity of DNA thereby lowering the limit of detection. Two more sensitive approaches would be to use qPCR, which allows precise quantification of the PCR product, or to use flow cytometry. These two techniques would give total viral abundance, although neither can distinguish between infectious and non-infectious particles. It would have been interesting to know the proportion of infectious OtVs being produced since this would have provided useful information on the virulence of the viruses. However, it is also possible that the number of non-infectious viruses was small, since selection would be greater for infectious viruses; if a virus particle was unable to infect a cell, it would subsequently be unable to replicate.

6.7 Incorporating host-virus population dynamics into climate change research

Viruses are now recognised for their importance in controlling populations of marine organisms, either directly through the mortality of their hosts, or indirectly by facilitating the availability of organic matter to organisms lower down the food web. We now know that environment can be one of the most important factors for explaining viral abundance (Finke *et al.*, 2017), and that viruses are important components of microbial communities (Suttle, 2007; Weitz and Wilhelm, 2012). Therefore, viruses must be considered in climate change research involving marine microorganisms. In their natural environment, organisms are adapting in response to

many changes simultaneously, both biotic and abiotic. Not only must algae adapt to physical changes in the oceans, but they must adapt in response to viruses, which are able to coevolve reciprocally.

Viruses are being increasingly incorporated into marine ecology studies, including those on climate change (Danovaro *et al.*, 2011). A study of *E. huxleyi* and EhV diversity in mesocosms showed that ocean acidification had different effects on host and virus diversities, and the authors called for all studies of ocean acidification effects on phytoplankton to include viruses (Highfield *et al.*, 2017). Marine virologists are currently carrying out large-scale sampling efforts to characterise the global ocean virome (viral metagenome) (Roux *et al.*, 2016). Alongside these studies that tell us so much about what is where, we must also consider how these viral communities and their hosts may be affected by environmental change. Not only should studies examine algal hosts on a species level, but it is also important to consider the resistance types of the individuals in a population (whether cells are susceptible or resistant to viral infection), to see whether they will respond to change in the same or different ways. This will provide information about the ways in which virus-host interactions and community compositions could change.

6.6 Concluding remarks

The work performed in this thesis is novel in three ways. First, for the first time I have measured the effects of more than one environmental change using an ecologically important species that has not been previously investigated. Second, I evolved susceptible and resistant hosts separately and directly compared their responses. This allows me to understand whether environment can affect susceptible and resistant hosts of the same species differently. Third, I applied two new techniques to examine RP *O. tauri* populations. A combination of liquid lysis assays to measure virus abundance and mathematical modelling have provided deeper insight into the population dynamics and epidemiology of this phenomenon that has been observed in laboratory cultures.

The results from this thesis show that viral resistance in microorganisms is complex. Resistance mechanisms and costs of resistance differ between species: bacteria generally acquire resistance through modifications of cell surface receptor proteins (Bohannan and Lenski, 2000), whereas eukaryotic algae display a range of intracellular resistance strategies (Bidle *et al.*, 2007; Frada *et al.*, 2008; Yau *et al.*, 2016). My results highlight the need for experiments that extend out from traditional model organisms and use ecologically relevant species. Since the range of resistance mechanisms and trade-offs is so diverse, there is still a lot to explore to understand whether environmental changes will affect susceptible and resistant hosts differently and how this could alter host-virus interactions and population densities in eukaryotic algae.

References

Abby, S., Touchon, M., de Jode, A., Grimsley, N., and Piganeau, G. (2014) Bacteria in Ostreococcus tauri cultures - friends, foes or hitchhikers? *Front. Microbiol.* **5**: 1–10.

Adams, J. and Rosenzweig, F. (2014) Experimental microbial evolution: history and conceptual underpinnings. *Genomics* **104**: 393–398.

Andersen, R.A. ed. (2005) Algal Culturing Techniques Elsevier Academic Press.

Atkinson, D., Ciotti, B.J., and Montagnes, D.J.S. (2003) Protists decrease in size linearly with temperature: ca. 2.5% degrees C(-1). *Proc. Biol. Sci.* **270**: 2605–11.

Avrani, S. and Lindell, D. (2015) Convergent evolution toward an improved growth rate and a reduced resistance range in Prochlorococcus strains resistant to phage. *Proc. Natl. Acad. Sci. U. S. A.* **112**: E2191-200.

Avrani, S., Schwartz, D. a., and Lindell, D. (2012) Virus-host swinging party in the oceans: Incorporating biological complexity into paradigms of antagonistic coexistence. *Mob. Genet. Elements* **2**: 88–95.

Avrani, S., Wurtzel, O., Sharon, I., Sorek, R., and Lindell, D. (2011) Genomic island variability facilitates Prochlorococcus-virus coexistence. *Nature* **474**: 604–8.

Bass, C., Denholm, I., Williamson, M.S., and Nauen, R. (2015) The global status of insect resistance to neonicotinoid insecticides. *Pestic. Biochem. Physiol.* **121**: 78–87.

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2014) Fitting Linear Mixed-Effects Models using lme4. *J. Stat. Softw.* **67**: 51.

Bell, G. (2008) Selection: The Mechanism of Evolution 2nd ed. Oxford University Press.

Bellec, L., Clerissi, C., Edern, R., Foulon, E., Simon, N., Grimsley, N., and Desdevises, Y. (2014) Cophylogenetic interactions between marine viruses and eukaryotic picophytoplankton. *BMC Evol. Biol.* **14**: 59.

Bellec, L., Grimsley, N., Derelle, E., Moreau, H., and Desdevises, Y. (2010) Abundance, spatial distribution and genetic diversity of Ostreococcus tauri viruses in two different environments. *Environ. Microbiol. Rep.* **2**: 313–21.

Bellec, L., Grimsley, N., and Desdevises, Y. (2010) Isolation of prasinoviruses of the green unicellular algae Ostreococcus spp. on a worldwide geographical scale. *Appl. Environ. Microbiol.* **76**: 96–101.

Bellec, L., Grimsley, N., Moreau, H., and Desdevises, Y. (2009) Phylogenetic analysis of new Prasinoviruses (Phycodnaviridae) that infect the green unicellular algae Ostreococcus, Bathycoccus and Micromonas. *Environ. Microbiol. Rep.* **1**: 114–23.

Bergh, O., Børsheim, K.Y., Bratbak, G., and Heldal, M. (1989) High abundance of viruses found in aquatic environments. *Nature* **340**: 467–468.

Bidle, K.D., Haramaty, L., Barcelos E Ramos, J., and Falkowski, P. (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophore, Emiliania huxleyi. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 6049–54.

Le Bihan, T., Martin, S.F., Chirnside, E.S., van Ooijen, G., Barrios-LLerena, M.E., O'Neill, J.S., et al. (2011) Shotgun proteomic analysis of the unicellular alga Ostreococcus tauri. *J. Proteomics* **74**: 2060–2070.

Björkman, J., Nagaev, I., Berg, O.G., Hughes, D., and Andersson, D.I. (2000) Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* **287**: 1479–82.

Blanc-Mathieu, R., Krasovec, M., Hebrard, M., Yau, S., Desgranges, E., Martin, J., et al. (2017) Population genomics of picophytoplankton unveils novel chromosome hypervariability. *Sci. Adv.*

Blanc-Mathieu, R., Verhelst, B., Derelle, E., Rombauts, S., Bouget, F.-Y., Carré, I., et al. (2014) An improved genome of the model marine alga Ostreococcus tauri unfolds by assessing Illumina de novo assemblies. *BMC Genomics* **15**: 1103.

Blower, S.M., Gershengorn, H.B., and Grant, R.M. (2000) A Tale of Two Futures: HIV and Antiretroviral Therapy in San Francisco. *Science (80-.).* **287**: 650–654.

Bohannan, B.J.M., Kerr, B., Jessup, C.M., Hughes, J.B., and Sandvik, G. (2002) Trade-offs and coexistence in microbial microcosms. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **81**: 107–115.

Bohannan, B.J.M. and Lenski, R.E. (2000) Linking genetic change to community evolution: Insights from studies of bacteria and bacteriophage. *Ecol. Lett.* **3**: 362–377.

Bohannan, B.J.M., Travisano, M., and Lenski, R.E. (1999) Epistatic Interactions Can Lower the Cost of Resistance to Multiple Consumers. *Evolution (N. Y).* **53**: 292–295.

Bohannan, B.J.M., Travisano, M., and Lenski, R.E. (2013) Epistatic Interactions Can Lower the Cost of Resistance to Multiple Consumers Author (s): Brendan J . M . Bohannan , Michael Travisano and Richard E . Lenski Published by : Society for the Study of Evolution Stable URL : http://www.jstor.org/stable/2640. **53**: 292–295.

Børsheim, K.Y. (1993) Native marine bacteriophages. *FEMS Microbiol. Lett.* **102**: 141–159.

Bratbak, G., Egge, J.K., and Heldal, M. (1993) Viral mortality of the marine alga Emiliania huxleyi (Haptophyceae) and termination of algal blooms. *Mar. Ecol. Prog. Ser.* **93**: 39–48.

Bratbak, G., Jacobsen, A., Heldal, M., Nagasaki, K., and Thingstad, F. (1998) Virus production in Phaeocystis pouchetii and its relation to host cell growth and nutrition. *Aquat. Microb. Ecol.* **16**: 1–9.

Brennan, G. and Collins, S. (2015) Growth responses of a green alga to multiple environmental drivers. *Nat. Clim. Chang.* **5**: 892–897.

Brockhurst, M.A., Chapman, T., King, K.C., Mank, J.E., Paterson, S., and Hurst, G.D.D. (2014) Running with the Red Queen: the role of biotic conflicts in evolution. *Proc. R. Soc. B Biol. Sci.* **281**: 20141382–20141382.

Brockhurst, M.A., Rainey, P.B., and Buckling, A. (2004) The effect of spatial heterogeneity and parasites on the evolution of host diversity. *Proc. Biol. Sci.* **271**: 107–11.

Brussaard, C.P.D. (2004) Viral Control of Phytoplankton Populations - A Review. *J. Eukaryot. Microbiol.* **51**: 125–138.

Brussaard, C.P.D., Bratbak, G., Baudoux, A.C., and Ruardij, P. (2007) Phaeocystis and its interaction with viruses. *Biogeochemistry* **83**: 201–215.

Buckling, A. and Rainey, P.B. (2002) Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. Biol. Sci.* **269**: 931–6.

Caldeira, K. and Wickett, M.E. (2003) Anthropogenic carbon and ocean pH. *Nature* **425**: 365.

Chen, F. and Suttle, C. (1995) Nested PCR with three highly degenerate primers for amplification and identification of DNA from related organisms. *Biotechniques* **18**: 609–611.

Chen, S., Gao, K., and Beardall, J. (2014) Viral attack exacerbates the susceptibility of a bloom-forming alga to ocean acidification. *Glob. Chang. Biol.* 629–636.

Clerissi, C., Desdevises, Y., and Grimsley, N. (2012) Prasinoviruses of the marine green alga Ostreococcus tauri are mainly species specific. *J. Virol.* **86**: 4611–9.

Clerissi, C., Grimsley, N., Ogata, H., Hingamp, P., Poulain, J., and Desdevisesa, Y. (2014) Unveiling of the diversity of prasinoviruses (Phycodnaviridae) in marine samples by using high-throughput sequencing analyses of PCR-amplified DNA polymerase and major capsid protein genes. *Appl. Environ. Microbiol.* **80**: 3150–3160.

Clerissi, C., Grimsley, N., Subirana, L., Maria, E., Oriol, L., Ogata, H., et al. (2014) Prasinovirus distribution in the Northwest Mediterranean Sea is affected by the environment and particularly by phosphate availability. *Virology* **466–467**: 146–157.

Corellou, F., Schwartz, C., Motta, J.-P., Djouani-Tahri, E.B., Sanchez, F., and Bouget, F.-Y. (2009) Clocks in the green lineage: comparative functional analysis of the circadian architecture of the picoeukaryote *Ostreococcus*. *Plant Cell* **21**: 3436–3449.

Countway, P.D. and Caron, D. a (2006) Abundance and Distribution of Ostreococcus sp. in the San Pedro Channel , California , as Revealed by Quantitative PCR. *Appl Env. Microbiol* **72**: 2496–2506.

Courties, C., Vaquer, A., Troussellier, M., Lautier, J., Chretiennot-Dinet, M.J., Jacques, N., et al. (1994) Smallest eukaryotic organism. *Nature* **370**: 255.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., and Smith, A.G. (2005) Algae acquire vitamin B12through a symbiotic relationship with bacteria. *Nature* **438**: 90–93.

Croft, M.T., Warren, M.J., and Smith, A.G. (2006) Algae need their vitamins. *Eukaryot. Cell* **5**: 1175–1183.

Danovaro, R., Corinaldesi, C., Dell'Anno, A., Fuhrman, J. a., Middelburg, J.J., Noble, R.T., and Suttle, C. a. (2011) Marine viruses and global climate change. *FEMS Microbiol. Rev.* **35**: 993–1034.

Dennehy, J.J. (2012) What Can Phages Tell Us about Host-Pathogen Coevolution? *Int J Evol Biol* **2012**: 396165.

Derelle, E., Ferraz, C., Escande, M.-L., Eychenié, S., Cooke, R., Piganeau, G., et al. (2008) Life-cycle and genome of OtV5, a large DNA virus of the pelagic marine unicellular green alga Ostreococcus tauri. *PLoS One* **3**: e2250.

Derelle, E., Ferraz, C., Rombauts, S., Rouzé, P., Worden, A.Z., Robbens, S., et al. (2006) Genome analysis of the smallest free-living eukaryote Ostreococcus tauri unveils many unique features. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 11647–11652.

Doney, S.C., Ruckelshaus, M., Emmett Duffy, J., Barry, J.P., Chan, F., English, C.A., et al. (2012) Climate Change Impacts on Marine Ecosystems. *Ann. Rev. Mar. Sci.* **4**: 11–37.

Elena, S.F. and Lenski, R.E. (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. **4**:.

Falkowski, P.G. (1998a) Biogeochemical Controls and Feedbacks on Ocean Primary Production. *Science (80-.).* **281**: 200–206.

Falkowski, P.G. (1998b) Biogeochemical Controls and Feedbacks on Ocean Primary Production. *Science (80-.).* **281**: 200–206.

Farinas, B., Mary, C., De O Manes, C.L., Bhaud, Y., Peaucellier, G., and Moreau, H. (2006) Natural synchronisation for the study of cell division in the green unicellular alga Ostreococcus tauri. *Plant Mol. Biol.* **60**: 277–292.

Field, C.B. (1998) Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science (80-.).* **281**: 237–240.

Finke, J.F., Hunt, B.P.V., Winter, C., Carmack, E.C., and Suttle, C.A. (2017) Nutrients and other environmental factors influence virus abundances across oxic and hypoxic marine environments. *Viruses* **9**: 1–15.

Finkel, Z. V., Beardall, J., Flynn, K.J., Quigg, A., Rees, T.A. V, and Raven, J.A. (2010) Phytoplankton in a changing world: Cell size and elemental stoichiometry. *J. Plankton Res.* **32**: 119–137.

Flint, S.J., Enquist, L.W., Krug, L., Racaniella, V., and Skalka, A. (2000) Principles of Virology: Molecular Biology, Pathogenesis, Virus Ecology 4th ed. ASM Press Washington, DC.

Frada, M., Probert, I., Allen, M.J., Wilson, W.H., and de Vargas, C. (2008) The "Cheshire Cat" escape strategy of the coccolithophore Emiliania huxleyi in response to viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 15944–9.

Frickel, J., Sieber, M., and Becks, L. (2016) Eco-evolutionary dynamics in a coevolving host-virus system. *Ecol. Lett.* **19**: 450–459.

Fuhrman, J.A. (1999) Marine viruses and their biogeochemical and ecological effects. *Nature* **399**: 541–8.

Geider, R., Platt, T., and Raven, J. (1986) Size dependence of growth and photosynthesis in diatoms: a synthesis. *Mar. Ecol. Ser.* **30**: 93–104.

Gómez, P. and Buckling, A. (2013) Coevolution with phages does not influence the evolution of bacterial mutation rates in soil. *ISME J.* **7**: 2242–2244.

Grimsley, N., Péquin, B., Bachy, C., Moreau, H., and Piganeau, G. (2010) Cryptic sex in the smallest eukaryotic marine green alga. *Mol. Biol. Evol.* **27**: 47–54.

Haaber, J. and Middelboe, M. (2009) Viral lysis of Phaeocystis pouchetii: implications for algal population dynamics and heterotrophic C, N and P cycling. *Isme J* **3**: 430–441.

Heath, S.E. and Collins, S. (2016) Mode of resistance to viral lysis affects host growth across multiple environments in the marine picoeukaryote Ostreococcus tauri. *Environ. Microbiol.* **doi:10.111**:

Heath, S.E., Knox, K., Vale, P.F., and Collins, S. (2017) Virus Resistance Is Not Costly in a Marine Alga Evolving under Multiple Environmental Stressors. *Viruses* **9**: 39.

Henderson, G.P., Gan, L., and Jensen, G.J. (2007) 3-D ultrastructure of O. tauri: electron cryotomography of an entire eukaryotic cell. *PLoS One* **2**: e749.

Highfield, A., Joint, I., Gilbert, J.A., Crawfurd, K.J., and Schroeder, D.C. (2017) Change in Emiliania huxleyi virus assemblage diversity but not in host genetic composition during an ocean acidification mesocosm experiment. *Viruses* **9**:.

Hurwitz, B.L. and Sullivan, M.B. (2013) The Pacific Ocean Virome (POV): A Marine Viral Metagenomic Dataset and Associated Protein Clusters for Quantitative Viral Ecology. *PLoS One* **8**: 1–12.

Iranzo, J., Krupovic, M., and Koonin, E. V (2016) The Double-Stranded DNA Virosphere as a Modular Hierarchical Network of Gene Sharing. **7**: 1–21.

Iyer, L.M., Aravind, L., and Koonin, E. V (2001) Common Origin of Four Diverse Families of Large Eukaryotic DNA Viruses. **75**: 11720–11734.

Iyer, L.M., Balaji, S., Koonin, E. V, and Aravind, L. (2006) Evolutionary genomics of nucleo-cytoplasmic large DNA viruses. *Virus Res.* **117**: 156–84.

Jacquet, S. and Bratbak, G. (2003) Effects of ultraviolet radiation on marine virus-phytoplankton interactions. *FEMS Microbiol. Ecol.* **44**: 279–289.

Jacquet, S., Heldal, M., Iglesias-Rodriguez, D., Larsen, A., Wilson, W., and Bratbak, G. (2002) Flow cytometric analysis of an Emiliana huxleyi bloom terminated by viral infection. *Aquat. Microb. Ecol.* **27**: 111–124.

Jessup, C.M. and Bohannan, B.J.M. (2008) The shape of an ecological trade-off varies with environment. *Ecol. Lett.* **11**: 947–959.

Karafistan, A., Martin, J.M., Rixen, M., and Beckers, J.M. (2002) Space and time distributions of phosphate in the Mediterranean Sea. *Deep. Res. I* **49**: 67–82.

Keller, M.D., Selvin, R.C., Claus, W., and Guillard, R.R.L. (1987) Media for the culture of oceanic ultraphytoplankton. **23**:.

Kermack, W. and McKendrick, A. (1927) A contribution to the mathematical theory of epidemics. *Proc. R. Soc. A* **115**: 700–721.

Klironomos, F.D., Berg, J., and Collins, S. (2013) How epigenetic mutations can affect genetic evolution: Model and mechanism. *BioEssays*.

Knowles, B., Silveira, C.B., Bailey, B. a, Barott, K., Cantu, V. a, Cobián-Güemes, a G., et al. (2016) Lytic to temperate switching of viral communities. *Nature* **531**: 466–70.

Koskella, B. and Brockhurst, M.A. (2014) Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* **38**: 916–931.

Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. (2015) lmerTest: Tests in Linear Mixed Effects Models. *Cran* **R package**: http://CRAN.R-project.org/package=lmerTest.

Larsen, J.B., Larsen, A., Thyrhaug, R., Bratbak, G., and Sandaa, R. -a. (2007) Response of marine viral populations to a nutrient induced phytoplankton bloom at different pCO2 levels. *Biogeosciences Discuss.* **4**: 3961–3985.

Larsen, J.B., Larsen, a., Thyrhaug, R., Bratbak, G., and Sandaa, R. -a. (2007) Marine viral populations detected during a nutrient induced phytoplankton bloom at elevated pCO_2 levels. *Biogeosciences Discuss.* **4**: 3961–3985.

Lazzari, P., Solidoro, C., Salon, S., and Bolzon, G. (2016) Spatial variability of phosphate and nitrate in the Mediterranean Sea: A modeling approach. *Deep. Res. Part I Oceanogr. Res. Pap.* **108**: 39–52.

Lennon, J.T., Khatana, S.A.M., Marston, M.F., and Martiny, J.B.H. (2007) Is there a cost of virus resistance in marine cyanobacteria? *ISME J.* **1**: 300–312.

Lenski, R.E. (1998) Bacterial evolution and the cost of antibiotic resistance. *Int. Microbiol.* **1**: 265–270.

Lenski, R.E. (1988) Dynamics of interactions between bacteria and virulent bacteriophage. *Adv. Microb. Ecol.* **10**: 1–44.

Lenski, R.E. (1988a) Experimental Studies of Pleiotropy and Epistasis in Escherichia coli. I. Variation in Competitive Fitness Among Mutants Resistant to Virus T4. *Evolution (N. Y)*. **42**: 425–432.

Lenski, R.E. (1988b) Experimental Studies of Pleiotropy and Epistasis in Escherichia coli . II . Compensation for Maldaptive Effects Associated with Resistance to Virus T4. *Evolution (N. Y)*. **42**: 433–440.

Lenski, R.E. and Levin, B.R. (1985) Constraints on the Coevolution of Bacteria and Virulent Phage: A Model, Some Experiments, and Predictions for Natural Communities. *Am. Nat.* **125**: 585–602.

León, M. and Bastías, R. (2015) Virulence reduction in bacteriophage resistant bacteria. *Front. Microbiol.* **6**: 1–7.

Levin, B.R., Stewart, F.M., and Chao, L. (1977) Resource-limited growth, competition and predation: A model and experimental studies with bacteria and bacteriophage. *Am. Nat.* **111**: 3–24.

Litchman, E., Steiner, D., and Bossard, P. (2003) Photosynthetic and growth responses of three freshwater algae to phosphorus limitation and daylength. *Freshw. Biol.* **48**: 2141–2148.

Lopez-Pascua, L.D.C. and Buckling, A. (2008) Increasing productivity accelerates host-parasite coevolution. *J. Evol. Biol.* **21**: 853–860.

Luria, S. and Delbrück, M. (1943) Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* **28**: 491–511.

Maat, D., Crawfurd, K., Timmermans, K., and Brussard, C. (2014) Elevated CO2 and phosphate limitation favor Micromonas pusilla through stimulated growth and reduced viral impact. *Appl. Environ. Microbiol.* 3119–3127.

Marston, M.F., Pierciey, F.J., Shepard, A., Gearin, G., Qi, J., Yandava, C., et al. (2012) Rapid diversification of coevolving marine Synechococcus and a virus. *PNAS* **109**: 4544–4549.

Martinez Martinez, J., Schroeder, D.C., Larsen, A., Bratbak, G., and Wilson, W.H. (2007) Molecular dynamics of Emiliania huxleyi and cooccurring viruses during two separate mesocosm studies. *Appl. Environ. Microbiol.* **73**: 554–562.

Martiny, J.B.H., Riemann, L., Marston, M.F., and Middelboe, M. (2014) Antagonistic coevolution of marine planktonic viruses and their hosts. *Ann Rev Mar Sci* **6**: 393–414.

McDaniel, L. and Paul, J.H. (2005) Effect of nutrient addition and environmental factors on prophage induction in natural populations of marine synechococcus species. *Appl. Environ. Microbiol.* **71**: 842–850.

McLachlan, J. (1961) The effect of salinity on growth and chlorophyll content in representative classes of unicellular marine algae. *Can. J. Microbiol.* **7**: 399–406.

Meaden, S., Paszkiewicz, K., and Koskella, B. (2015) The cost of phage resistance in a plant pathogenic bacterium is context-dependent. *Evolution (N. Y).* **69**: 1321–1328.

Melnyk, A.H., Wong, A., and Kassen, R. (2015) The fitness costs of antibiotic resistance mutations. *Evol. Appl.* **8**: 273–283.

Menge, D.N.L. and Weitz, J.S. (2009) Dangerous nutrients: Evolution of phytoplankton resource uptake subject to virus attack. *J. Theor. Biol.* **257**: 104–115.

Meyer, J.R., Agrawal, A.A., Quick, R.T., Dobias, D.T., Schneider, D., and Lenski, R.E. (2010) Parallel changes in host resistance to viral infection during 45,000 generations of relaxed selection. *Evolution (N. Y).* **64**: 3024–3034.

Middelboe, M. (2000) Bacterial growth rate and marine virus-host dynamics. *Microb. Ecol.* **40**: 114–124.

Monier, A., Welsh, R.M., Gentemann, C., Weinstock, G., Sodergren, E., Armbrust, E.V., et al. (2012) Phosphate transporters in marine phytoplankton and their viruses: Cross-domain commonalities in viral-host gene exchanges. *Environ. Microbiol.* **14**: 162–176.

Morán, X.A.G., López-Urrutia, Á., Calvo-Díaz, A., and Li, W.K.W. (2010) Increasing importance of small phytoplankton in a warmer ocean. *Glob. Chang. Biol.* **16**: 1137–1144.

Moreira, D. and López-García, P. (2009) Ten reasons to exclude viruses from the tree of life. *Nat. Rev. Microbiol.* **7**: 306–311.

Motegi, C., Kaiser, K., Benner, R., and Weinbauer, M.G. (2015) Effect of P-limitation on prokaryotic and viral production in surface waters of the Northwestern Mediterranean Sea. *J. Plankton Res.* **37**: 16–20.

Moulager, M., Monnier, A., Jesson, B., Bouvet, R., Mosser, J., Schwartz, C., et al. (2007) Light-dependent regulation of cell division in Ostreococcus: evidence for a major transcriptional input. *Plant Physiol.* **144**: 1360–9.

Nagasaki, K. and Yamaguchi, M. (1998) Effect of temperature on the algicidal activity and the stability of HaV (Heterosigma akashiwo virus). *Aquat. Microb. Ecol.* **15**: 211–216.

Northfield, T.D. and Ives, A.R. (2013) Coevolution and the Effects of Climate Change on Interacting Species. *PLoS Biol.* **11**:.

O'Kelly, C.J., Sieracki, M.E., Thier, E.C., and Hobson, I.C. (2003) A transient bloom of Ostreococcus (Chlorophyta, Prasinophyceae_ in West NEck Bay, Long Island, New York. *J. Phy* **39**: 850–854.

Pal, C., Maciá, M.D., Oliver, A., Schachar, I., and Buckling, A. (2007) Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* **450**: 1079–1081.

Parsons, R.J., Breitbart, M., Lomas, M.W., and Carlson, C.A. (2012) Ocean time-series reveals recurring seasonal patterns of virioplankton dynamics in the northwestern Sargasso Sea. *ISME J.* **6**: 273–284.

Peter, K.H. and Sommer, U. (2015) Interactive effect of warming, nitrogen and phosphorus limitation on phytoplankton cell size. *Ecol. Evol.* **5**: 1011–1024.

Phillips, A.N. (1997) Reduction of HIV Concentration during Acute Infection : Independence from a Specific Immune Response Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2890152 Reduction of HIV Concentration During. *Science (80-.).* **271**: 497–499.

Raven, J.A. (1998) The twelfth Tansley Lecture. Small is beautiful: The picophytoplankton. *Funct. Ecol.* **12**: 503–513.

Renk, H. and Ochocki, S. (1998) Photosynthetic rate and light curves of phytoplankton in the southern Baltic. *Oceanologia* **40**: 331–344.

Riemann, B., Simonsen, P., and Stensgaard, L. (1989) The carbon and chlorophyll content of phytoplankton from various nutrient regimes. *J. Plankton Res.* **11**: 1037–1045.

Robbens, S., Khadaroo, B., Camasses, A., Derelle, E., Ferraz, C., Inzé, D., et al. (2005) Genome-wide analysis of core cell cycle genes in the unicellular green alga Ostreococcus tauri. *Mol. Biol. Evol.* **22**: 589–597.

Roux, S., Brum, J.R., Dutilh, B.E., Sunagawa, S., Duhaime, M.B., Loy, A., et al. (2016) Ecogenomics and biogeochemical impacts of uncultivated globally abundant ocean viruses. *Nature* **537**: 689–693.

Ruiz, E., Oosterhof, M., Sandaa, R.A., Larsen, A., and Pagarete, A. (2017) Emerging interaction patterns in the Emiliania huxleyi-EhV system. *Viruses* **9**:.

Ryther, J. and Menzel, D. (1959) Light adaptation by marine phytoplankton. *Limnol. Ocean.*

Sandaa, R.A., Gómez-Consarnau, L., Pinhassi, J., Riemann, L., Malits, A., Weinbauer, M.G., et al. (2009) Viral control of bacterial biodiversity - Evidence from a nutrient-enriched marine mesocosm experiment. *Environ. Microbiol.* **11**: 2585–2597.

Schaum, C.-E., Rost, B., and Collins, S. (2015) Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. *ISME J.* 1–10.

Schaum, E. and Collins, S. (2014) Plasticity predicts evolution in a marine alga. *Proc. R. Soc. B* **281**: 20141486.

Schaum, E., Rost, B., Millar, A.J., and Collins, S. (2012) Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. *Nat. Clim. Chang.* **3**: 298–302.

Schroeder, D.C., Oke, J., Hall, M., Malin, G., and Wilson, W.H. (2003) Viral succession observed during an Emiliania huxleyi bloom. *Appl. Environ. Microbiol.* **69**: 2484–2490.

Seed, K.D., Faruque, S.M., Mekalanos, J.J., Calderwood, S.B., Qadri, F., and Camilli, A. (2012) Phase Variable O Antigen Biosynthetic Genes Control Expression of the Major Protective Antigen and Bacteriophage Receptor in Vibrio cholerae O1. *PLoS Pathog.* **8**:.

Seymour, J.R., Amin, S.A., Raina, J.B., and Stocker, R. (2017) Zooming in on the phycosphere: The ecological interface for phytoplankton-bacteria relationships. *Nat. Microbiol.* **2**:.

Sharon, I., Battchikova, N., Aro, E.-M., Giglione, C., Meinnel, T., Glaser, F., et al. (2011) Comparative metagenomics of microbial traits within oceanic viral communities. *ISME J.* **5**: 1178–1190.

Short, S.M. (2012) The ecology of viruses that infect eukaryotic algae. *Environ. Microbiol.* **14**: 2253–71.

Sigaud, T.C.S. and Aidar, E. (1993) Salinity and temperature effects on the growth and chlorophyll-a content of some planktonic aigae. *Bol. do Inst. Ocean.* **41**: 95–103.

Spilling, K., Ylöstalo, P., Simis, S., and Seppälä, J. (2015) Interaction effects of light, temperature and nutrient limitations (N, P and Si) on growth, stoichiometry and photosynthetic parameters of the cold-water diatom Chaetoceros wighamii. *PLoS One* **10**: 1–18.

Stoddard, L.I., Martiny, J.B.H., and Marston, M.F. (2007) Selection and characterization of cyanophage resistance in marine Synechococcus strains. *Appl. Environ. Microbiol.* **73**: 5516–5522.

Sullivan, M.B., Coleman, M.L., Weigele, P., Rohwer, F., and Chisholm, S.W. (2005) Three Prochlorococcus cyanophage genomes: Signature features and ecological interpretations. *PLoS Biol.* **3**: 0790–0806.

Šupraha, L., Gerecht, A.C., Probert, I., and Henderiks, J. (2015) Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation. *Sci. Rep.* **5**: 16499.

Suttle, C.A. (2007) Marine viruses--major players in the global ecosystem. *Nat. Rev. Microbiol.* **5**: 801–12.

Suttle, C.A. (2005) Viruses in the sea. *Nature* **437**: 356–61.

Suttle, C.A. and Chan, A.M. (1993) Marine cyanophages infecting oceanic and coastal strains of Synechococcus: abundance, morphology, cross-infectivity and growth characteristics. *Mar. Ecol. Prog. Ser.* **92**: 99–109.

Tarutani, K., Nagasaki, K., and Yamaguchi, M. (2006) Virus adsorption process determines virus susceptibility in Heterosigma akashiwo (Raphidophyceae). *Aquat. Microb. Ecol.* **42**: 209–213.

Thingstad, T.F. (2000) Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol. Oceanogr.* **45**: 1320–1328.

Thomas, R., Grimsley, N., Escande, M.-L., Subirana, L., Derelle, E., and Moreau, H. (2011) Acquisition and maintenance of resistance to viruses in eukaryotic phytoplankton populations. *Environ. Microbiol.* **13**: 1412–20.

Thyrhaug, R., Larsen, A., Brussaard, C.P.D., and Mcfadden, P. (2002) Cell Cycle Dependent Virus Production in Marine Phytoplankton 1. *Cell Cycle* **343**: 338–343.

Tuomi, P., Fagerbakke, K.M., Bratbak, G., and Heldal, M. (1995) Nutritional enrichment of a microbial community - the effects on activity, elemental composition, community structure and virus production. *FEMS Microbiol. Ecol.* **16**: 123–134.

Viprey, M., Guillou, L., Ferreol, M., and Vaulot, D. (2008) Wide genetic diversity of picoplanktonic green algae (Chloroplastida) in the Mediterranean Sea uncovered by a phylum-biased PCR approach. *Environ. Microbiol.* **10**: 1804–1822.

Weinbauer, M.G. (2004) Ecology of prokaryotic viruses. **28**: 127–181.

Weitz, J.S., Stock, C.A., Wilhelm, S.W., Bourouiba, L., Coleman, M.L., Buchan, A., et al. (2015) A multitrophic model to quantify the effects of marine viruses on microbial food webs and ecosystem processes. *ISME J.* **9**: 1352–1364.

Weitz, J.S. and Wilhelm, S.W. (2012) Ocean viruses and their effects on microbial communities and biogeochemical cycles. *F1000 Biol. Rep.* **4**: 17.

Wells, L.E. and Deming, J.W. (2006) Effects of temperature, salinity and clay particles on inactivation and decay of cold-active marine Bacteriophage 9A. *Aquat. Microb. Ecol.* **45**: 31–39.

Weynberg, K.D., Allen, M.J., Ashelford, K., Scanlan, D.J., and Wilson, W.H. (2009) From small hosts come big viruses: the complete genome of a second Ostreococcus tauri virus, OtV-1. *Environ. Microbiol.* **11**: 2821–39.

Weynberg, K.D., Allen, M.J., Gilg, I.C., Scanlan, D.J., and Wilson, W.H. (2011) Genome sequence of Ostreococcus tauri virus OtV-2 throws light on the role of picoeukaryote niche separation in the ocean. *J. Virol.* **85**: 4520–9.

Wigington, C.H., Sonderegger, D., Brussaard, C.P.D., Buchan, A., Finke, J.F., Fuhrman, J.A., et al. (2016) Re-examination of the relationship between marine virus and microbial cell abundances. *Nat. Microbiol.* **1**: 15024.

Wilhelm, S.W. and Suttle, C.A. (1999) Viruses and nutrient cycles in the sea. *Bioscience* **49**: 781–788.

Williamson, S.J., Houchin, L. a, Mcdaniel, L., and Paul, J.H. (2002) Seasonal Variation in Lysogeny as Depicted by Prophage Induction in Tampa Bay , Seasonal Variation in Lysogeny as Depicted by Prophage Induction in Tampa Bay , Florida. *Appl. Environ. Microbiol.* **68**: 4307–4314.

Wilson, W.H., Carr, N.G., and Mann, N.H. (1996) The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium Synechococcus sp. WH7803. *J. Phycol.* **32**: 506–516.

Wilson, W.H. and Mann, N.H. (1997) Lysogenic and lytic viral production in marine microbial communities. *Aquat. Microb. Ecol.* **13**: 95–100.

Wilson, W.H., Schroeder, D.C., Allen, M.J., Holden, M.T.G., Parkhill, J., Barrell, B.G., et al. (2005) Complete genome sequence and lytic phase transcription profile of a Coccolithovirus. *Science* **309**: 1090–2.

Wilson, W.H., Turner, S., and Mann, N.H. (1998) Population Dynamics of Phytoplankton and Viruses in a Phosphate-limited Mesocosm and their Effect on DMSP and DMS Production. *Estuar. Coast. Shelf Sci.* **46**: 49–59.

Wommack, K.E. and Colwell, R.R. (2000) Virioplankton: Viruses in Aquatic Ecosystems. *Microbiol. Mol. Biol. Rev.* **64**: 69–114.

Worden, A.Z., Nolan, J.K., and Palenik, B. (2004) Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. *Limnol. Oceanogr.* **49**: 168–179.

Wozniak, B., Hapter, R., and Dera, J. (1989) Light curves of marine plankton photosynthesis in the Baltic *. *Oceanologia* **29**: 61–78.

Yau, S., Hemon, C., Derelle, E., Moreau, H., Piganeau, G., and Grimsley, N. (2016) A Viral Immunity Chromosome in the Marine Picoeukaryote, Ostreococcus tauri. *PLoS Pathog.* **12**: e1005965.
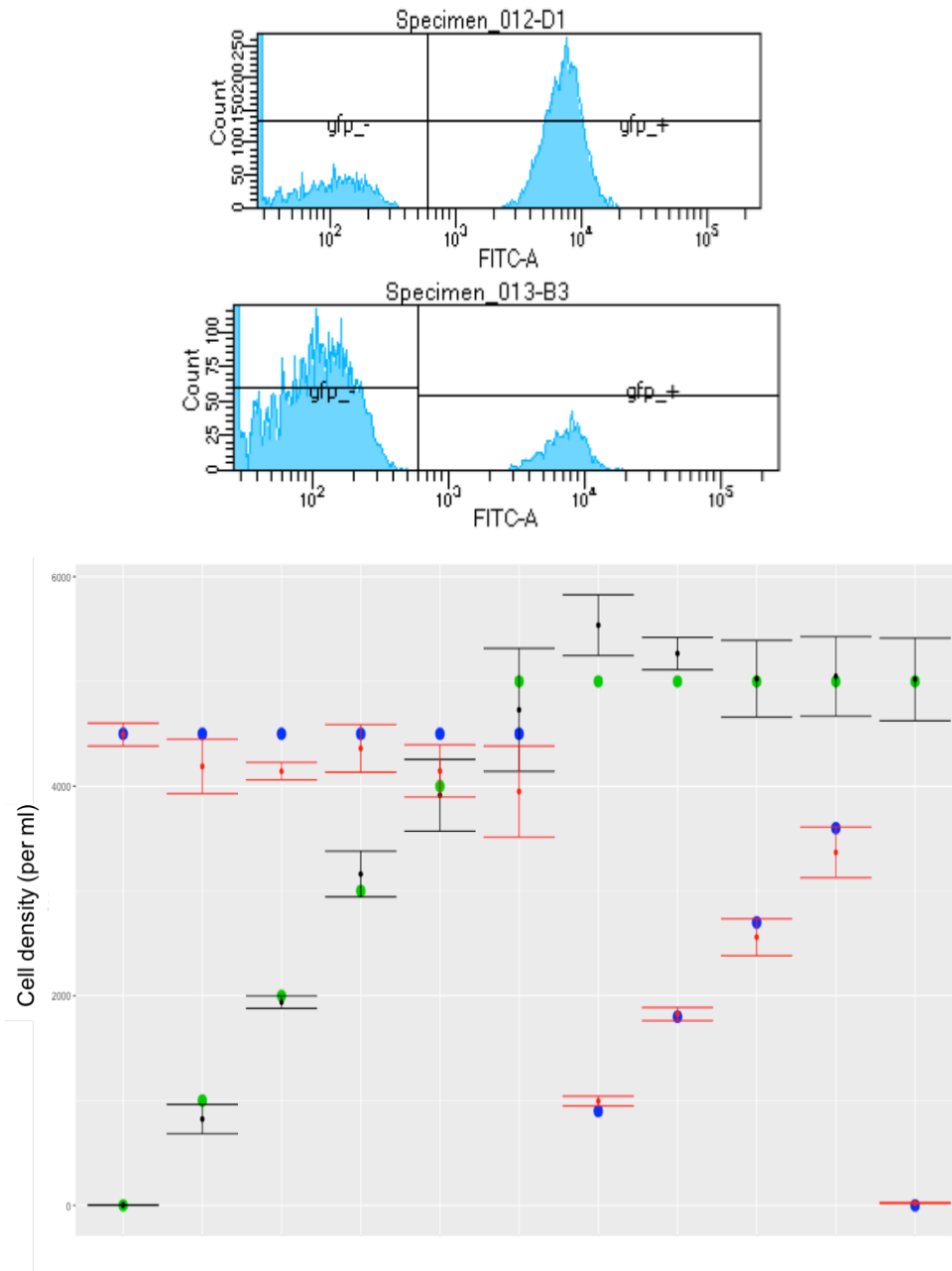
Appendix figure 1. The top panels show examples of FITC gates used to measure populations of GFP negative and GFP positive cells. To test that the two populations (GFP and RCC4221) could be distinguished without overlap, I mixed different proportions of each population at known densities. GFP observed (red), GFP expected (blue), RCC4221 observed (black), and RCC4221 expected (green).
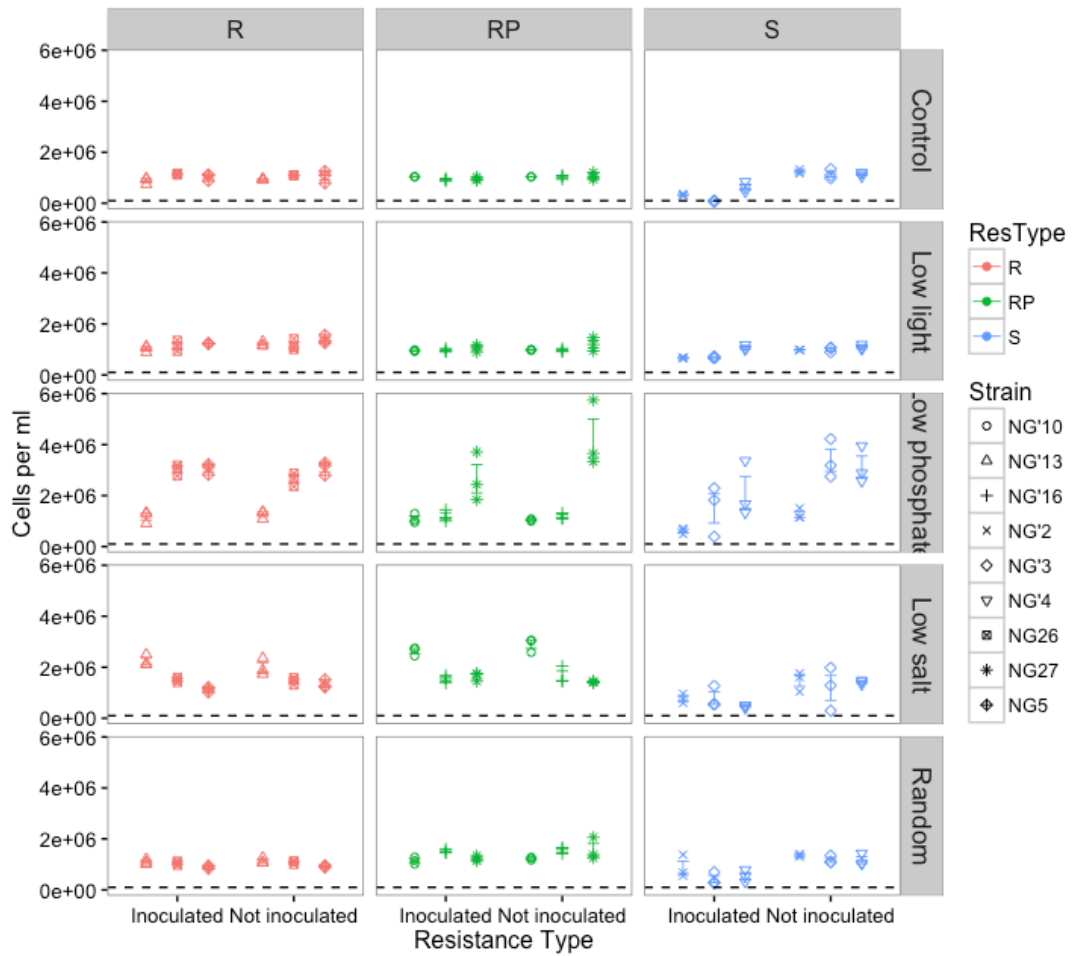
Appendix figure 2. Mean (± SE) cell density ml^{-1} of resistant (R), resistant producer (RP) and susceptible (S) *O. tauri* lines 7 days after OtV5 inoculation in five environments. Points represents the average of the three assay replicates for each evolved population. Inoculated = populations inoculated with OtV5, Not inoculated = negative control populations that were grown for the same period without OtV5 inoculation. There were three evolved populations of each line. The dashed line represents the starting cell density at 100 000 cell ml^{-1}.
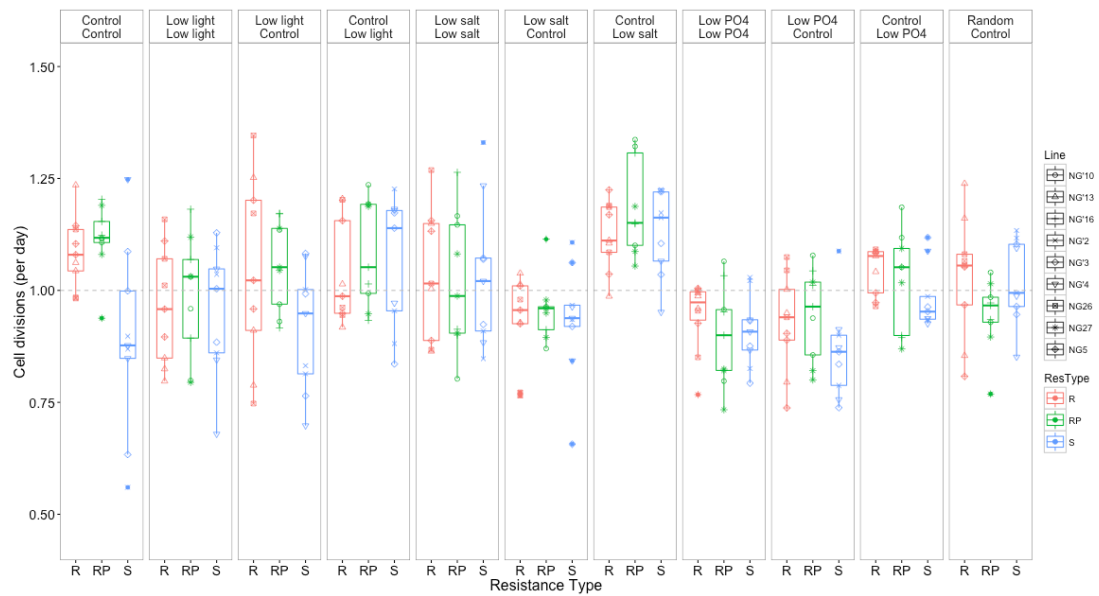
Appendix figure 3. Mean *O. tauri* cell divisions per day (±SEM) showing direct and correlated responses. R = resistant, RP = resistant producer, S = susceptible. Each panel represents a growth assay, with populations evolved in the selection environment (top label) and growth rates measured in the assay environment (bottom label). The dashed line indicates, for reference, one cell division per day.
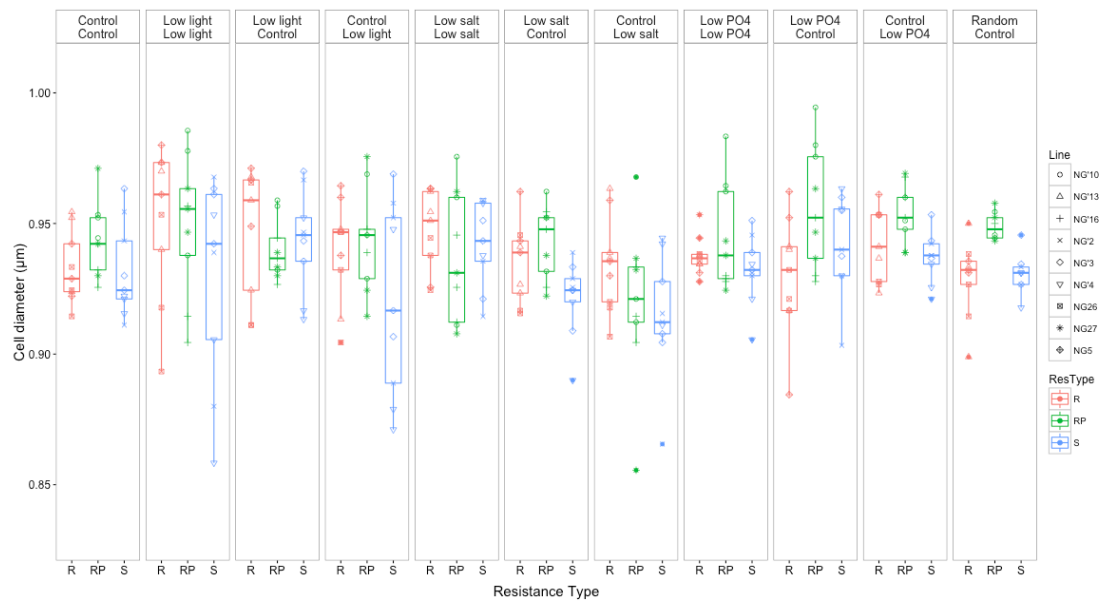
Appendix figure 4. Mean cell size of *O. tauri* populations evolved and assayed under different environments showing the direct and correlated response to evolution. R = resistant, RP = resistant producer, S = susceptible. Each panel represents an assay, with populations evolved in the selection environment (top label) and cell size measured in the assay environment (bottom label).
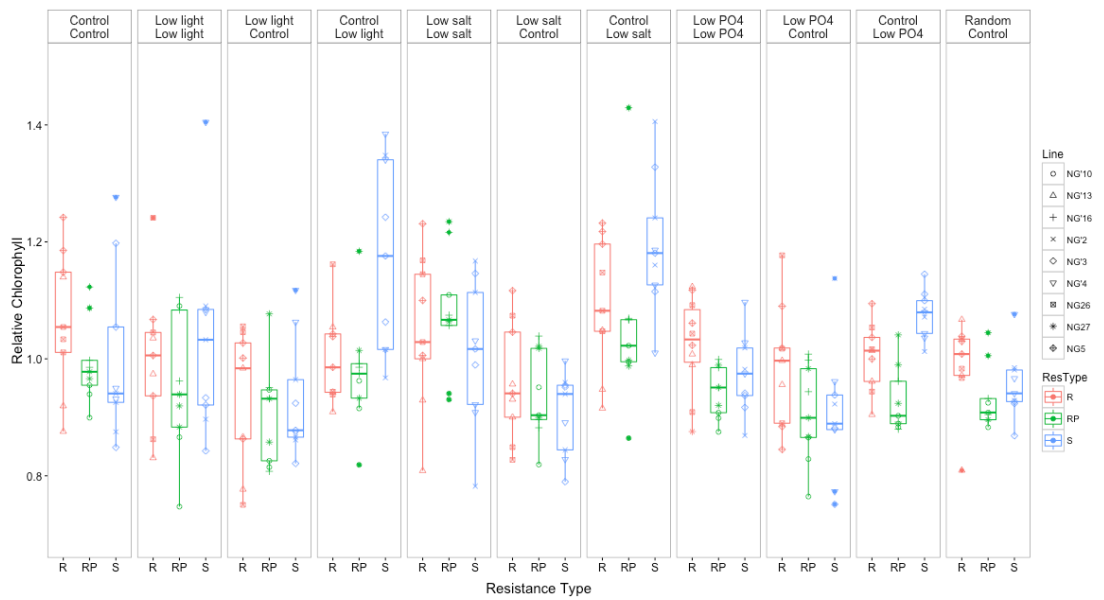
Appendix figure 5. Relative chlorophyll content per cell volume of *O. tauri* populations evolved and assayed under different environments showing direct and correlated response to evolution. R = resistant, RP = resistant producer, S = susceptible. Each panel represents an assay, with populations evolved in the selection environment (top label) and mean chlorophyll content per cell measured in the assay environment (bottom label).
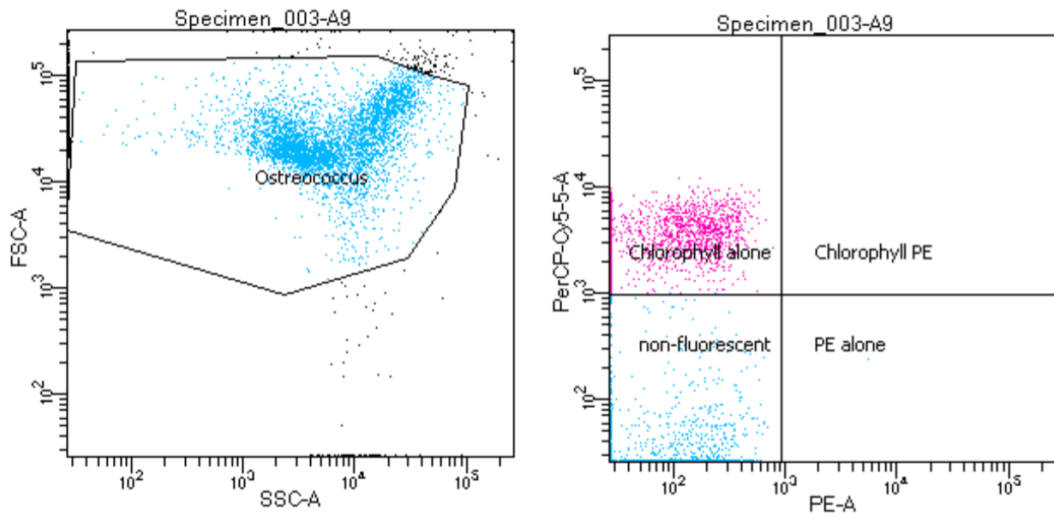
Appendix figure 6. Dot plot of flow cytometry analysis of a laboratory *O. tauri* culture. Each dot represents a single cell analysed by flow cytometry. The first image shows all particles analysed by the flow cytometer that fell within the 1μm size range for *O. tauri* as measured by size (forward scatter; FSC-A) and granularity (side scatter; SSC-A). Within this region of interest, the second frame shows the proportion of particles that contained chlorophyll (shown in pink as measured by PerCP-Cy5-5-A fluorescence) indicating a healthy *O. tauri* population, and a smaller proportion that did not contain chlorophyll. This indicates that cells of the same size, probably bacteria, could have been present.

This script can be run using R.

```r
rm(list=ls()) # clear R's brain
library(deSolve) # load package


##### RP MODEL #####


parms<- c(beta=0.35, #adsorption rate 35%
          d=0.05, #natural death rate
          b=1, #birth rate- 1 division per day
          gamma=0.01, #virus decay rate
          r=0.3, # rate RP goes to R
          c=3, # virus production from RP
          k=1000000, # carrying capacity
          D=0.01) # dilution rate
y<- c(RP=100, R=0, V=0)
t<- seq(0, 500, by=1)
mod<- function(t, y, parms) {
  with(as.list(c(y, parms)), {
    dRP <- b*RP*(1-(RP+R)/k) - d*RP -r*RP
    dR <- r*RP + b*R*(1-(R+RP)/k) - d*R
    dV <- c*RP - beta*RP*V - beta*R*V - gamma*V
    return(list(c(dRP, dR, dV)))
  })
}
etime<- seq(0,500,7)
eventfun<- function(t, y, parms) {
  with(as.list(c(y, parms)), {
    return(c(RP*D, R*D, V*D))
  })
}
out<- ode(y, t, mod, parms,
          events=list(func=eventfun, time=etime))
plot(out)
plot(out, ylim=c(0,1000000))


##### SIRV MODEL #####


parms<- c(beta=0.35, #adsorption rate- 20-35%
          lambda=0.99, #lysis rate
          d=0.05, #natural death rate
          b=1, #birth rate- 1 division per day
          gamma=0.01,#virus decay rate
```

```r
        alpha=0.01, # rate I goes to R
        k=1000000,# carrying capacity
        D=0.01) # dilution rate
y<- c(S=1000, I=0, R=1000000, V=20020) # 50x as many cells as V
t<- seq(0, 500, by=1)
mod<- function(t, y, parms) {
  with(as.list(c(y, parms)), {
    dS <- b*S*(1-(S+I+R)/k) - beta*S*V - d*S
     dI <- beta*S*V - lambda*I - alpha*I + b*I*(1-(S+I+R)/k)
     dR <- alpha*I + b*R*(1-(S+I+R)/k) - d*R
     dV <- lambda*I*25 - beta*S*V - beta*R*V- gamma*V
     return(list(c(dS, dI, dR, dV)))
  })
}
etime<- seq(0,500,7)
eventfun<- function(t, y, parms) {
  with(as.list(c(y, parms)), {
    return(c(S*D, I*D, R*D, V*D))
  })
}
out<- ode(y, t, mod, parms,
         events=list(func=eventfun, time=etime))
plot(out)
plot(out, ylim=c(0,1000000))
```

Mode of resistance to viral lysis affects host growth across multiple environments in the marine picoeukaryote *Ostreococcus tauri*

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Summary

Viruses play important roles in population dynamics and as drivers of evolution in single-celled marine phytoplankton. Viral infection of *Ostreococcus tauri* often causes cell lysis, but two spontaneously arising resistance mechanisms occur: resistant cells that cannot become infected and resistant producer cells that are infected but not lysed, and which may slowly release viruses. As of yet, little is known about how consistent the effects of viruses on their hosts are across different environments. To measure the effect of host resistance on host growth, and to determine whether this effect is environmentally dependent, we compared the growth and survival of susceptible, resistant and resistant producer *O. tauri* cells under five environmental conditions with and without exposure to *O. tauri* virus. While the effects of exposure to virus on growth rates did not show a consistent pattern in populations of resistant cells, there were several cases where exposure to virus affected growth in resistant hosts, sometimes positively. In the absence of virus, there was no detectable cost of resistance in any environment, as measured by growth rate. In fact, the opposite was the case, with populations of resistant producer cells having the highest growth rates across four of the five environments.

Introduction

Marine viruses play a large role in nutrient and energy cycling in the oceans. Viral lysis of single celled organisms releases large quantities of organic matter into the

environment, making nutrients available for use by bacteria and algae. This process has been termed the viral shunt (Wilhelm and Suttle, 1999). Studies on marine viruses typically focus on the importance of viruses in nutrient cycling and the release of organic matter through cell lysis. Despite the important role of marine viruses in ecosystem function across many environments, from nutrient rich coastal waters to more oligotrophic regions of the open ocean (Bruussard, 2004), host–virus interactions are typically studied in single environments. Here, we use the *Ostreococcus tauri*/*Ostreococcus tauri* virus model system to investigate variation in host–virus interactions across environments to understand (i) whether susceptibility/resistance to viruses changes with environmental change and (ii) whether the growth effect of host resistance depends on environmental context or resistance type.

We explore the relationship between host responses to environmental change and the resistance strategies of those hosts using the marine picoeukaryote *Ostreococcus tauri* (order Mamiellales). *O. tauri* is commonly isolated from Mediterranean lagoons that are connected to the open ocean via narrow channels (Clerissi *et al.*, 2014). These channels limit the exchange of seawater between the lagoon and ocean, making variations in the environmental salinity, pH, temperature and nutrients more extreme than in the open ocean (Bellec *et al.*, 2010; Clerissi *et al.*, 2014). *Ostreococcus tauri* viruses (OtVs) have been sampled frequently in water samples collected from lagoon and coastal waters where *O. tauri* is found. OtVs have strict host specificity (Clerissi *et al.*, 2012), and the three OtVs sequenced to date have all been described as lytic viruses (Derelle *et al.*, 2008; Weynberg *et al.*, 2009; 2011). Virus infection of *O. tauri* usually causes cell lysis in susceptible (S) cells, though two mechanisms of resistance have been observed (Thomas *et al.*, 2011). In the first case, viruses are unable to infect the host, and these cells are referred to here as resistant (R). In the second case, hosts are tolerant to viral infection and are able to slowly release them without damage to the host cell. These cells are termed resistant producers (RP). In this paper, we refer to the three cell types as resistance types.

Resistance type could have consequences for growth and other cell properties, such as size and chlorophyll content. For example, a trade-off of acquiring resistance to viral lysis may come as a fitness cost. This often occurs as reduced competitive ability (Lenski, 1988; Bohannan *et al.*, 2002) and sometimes reduced growth rate (Lennon *et al.*, 2007; Frickel *et al.*, 2016). A modification in cell surface receptors to limit virus attachment could also result in a loss of the original function of the protein, such as metabolism or being able to target the host immune system. In several bacteria species, loss of a bacteriophage receptor results in lower virulence of the bacteria in its host, thereby lowering the fitness of resistant compared to non-resistant strains (Seed *et al.*, 2012; León and Bastías, 2015). Lastly, strong resistance to one specific virus strain may lead to increased susceptibility to lysis by other strains, as has been observed in *O. tauri* (Clerissi *et al.*, 2012) and cyanobacteria (Marston *et al.*, 2012; Avrani and Lindell, 2015).

The group of viruses that infects phytoplankton is the Phycodnaviruses. These viruses have been studied under environmental conditions that differ from a benign control environment in a single driver, such as increases in temperature (Nagasaki and Yamaguchi, 1998; Wells and Deming, 2006), nutrient (Bratbak *et al.*, 1993; 1998; Bellec *et al.*, 2010; Clerissi *et al.*, 2014), light (Bratbak *et al.*, 1998; Weinbauer, 2004), UV (Jacquet and Bratbak, 2003), CO_2 (Larsen *et al.*, 2007; Chen *et al.*, 2014; Maat *et al.*, 2014) and pH levels (Weinbauer, 2004). When environmental conditions are stressful, one consequence can be inactivation of the virus particle. This affects host–virus interactions by preventing infection through structural degradation, the inability of the virus to inject its genome into the host or the inability of the virus to replicate (Børsheim, 1993; Jacquet and Bratbak, 2003). Additionally, since viral replication and life cycle are often closely linked to host metabolism, environmental changes such as increased temperature or nutrients will often have an indirect effect on responses to viral attack (Weinbauer, 2004; Danovaro *et al.*, 2011). Understanding the role of viruses in marine communities requires investigating their activity across environments. Here, we focus on the environmental changes of increased temperature, decreased nutrients, decreased light and decreased salinity levels.

Previous studies of resistance in *O. tauri* found that when each resistance type was maintained separately there was no significant difference in growth rates, such that a cost of resistance was too low to be detected by differences in growth alone. However, when resistant types were competed against each other, a competitive hierarchy was observed in which S had the fastest growth rate, followed by R and then by RP (Thomas *et al.*, 2011). Since the three resistance types share the same starting genotype, it is possible to make direct comparisons between them. In this study, an experiment was performed in which three populations of each *O. tauri* resistance type (S, R and RP) derived from a common ancestor were grown for 1 week in the following environments in both the absence and presence of OtV5: high temperature, low light, low phosphate and low salt. These environments were selected to represent relatively small variations from the control environment in which the populations are normally maintained in the laboratory, so that the cells responded, but were still able to grow at a rate that was measureable. The average number of cell divisions per day over a single transfer cycle (7 days), cell size and cell chlorophyll content were measured in the novel environments in the absence of OtV5. Offspring production over a fixed period of time is a proxy for fitness in single celled organisms in batch culture experiments (Brennan and Collins, 2015). Cell size and chlorophyll content were measured as additional phenotypes, to examine effects on organismal function other than cell division rates, since only small differences in growth were detected previously (Thomas *et al.*, 2011). After 1 week of growth in the novel environment, all populations were inoculated with OtV5 and cell densities were measured three days after inoculation to test for susceptibility to viral lysis.

Results

The effect of viral exposure on cell division rates depends on resistance type

After 1 week of growth in a novel environment, all populations were inoculated with OtV5 and cell densities were measured three days later. Supporting Information Tables S1 and S2 provide all statistical outputs in this study. Susceptibility of *O. tauri* to OtV5 was driven by resistance type, as expected (ANOVA, resistance type $\times$ virus treatment, $F_{2,234} = 360.14$, $p < 0.0001$). After inoculation of *O. tauri* with OtV5, all R and RP cells remained resistant to lysis and S cells remained susceptible (Fig. 1). Thus, OtV5 inoculation had a significant effect on cell density (ANOVA effect of virus treatment on growth, $F_{1,234} = 361.62$, $p < 0.0001$), since populations of S cells fell to almost zero (Fig. 1). No difference was observed in resistance between R and RP populations ($t = 0.46$, $p = 0.66$).

Counter to our expectation, the effect of virus inoculation did not vary with environment (ANOVA, environment $\times$ virus treatment, $F_{4,234} = 0.89$, $p < 0.46$). However, environment alone had a significant effect on growth ($F_{4,234} = 26.01$, $p < 0.0001$), because of the S cell lysis in all environments. Additionally, an interaction was identified between resistance type and environment (ANOVA, environment $\times$ resistance type, $F_{8,234} = 6.09$, $p < 0.0001$). For both R and RP cells, there were cases where virus inoculation resulted in higher growth rates than the non-inoculated controls (Fig. 1). Cell densities were repeatedly higher in one inoculated population (NG′13) than the control in the low salt environment in R cells and in the low light environment for one population (NG27) in RP cells. This indicates
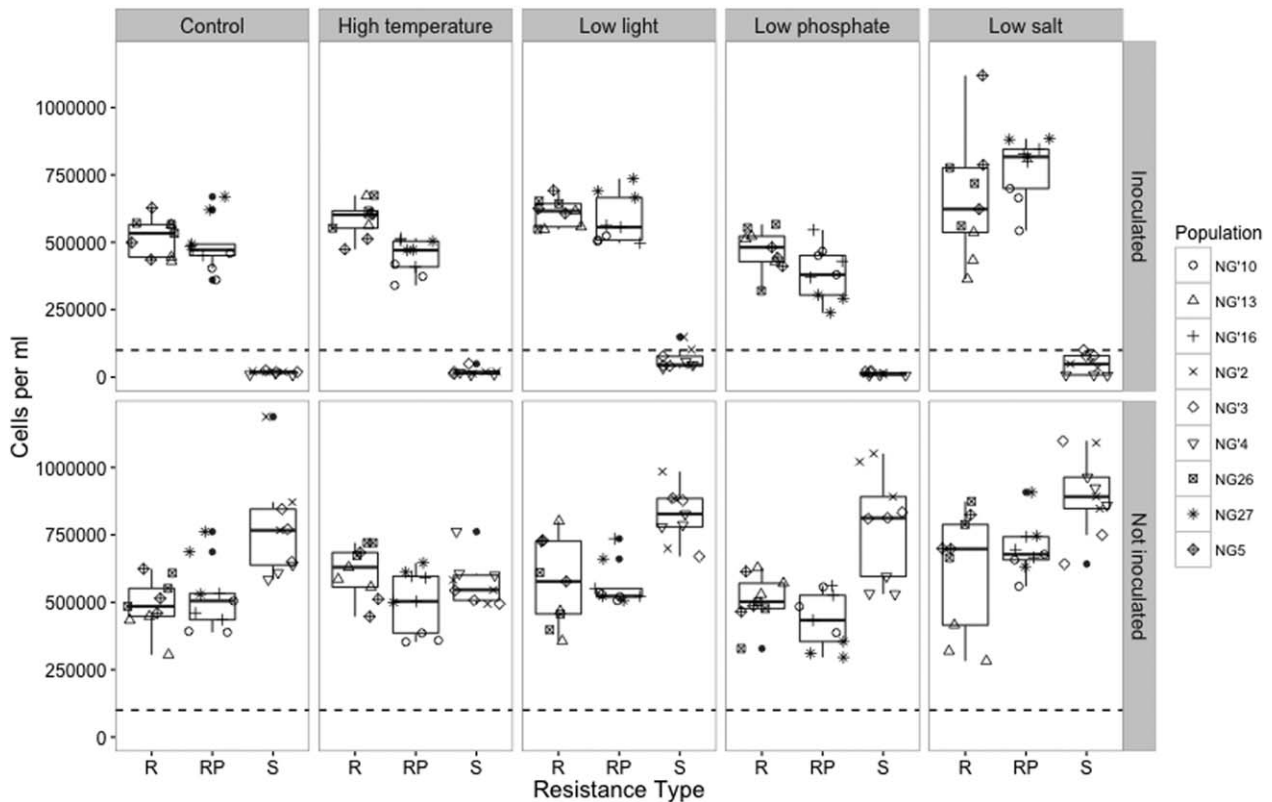
Fig. 1. Mean cell densities ml^{-1} of resistant (R), resistant producer (RP) and susceptible (S) *O. tauri* cells. Inoculated = cells inoculated with OtV5, Not inoculated = control cultures that were grown for the same amount of time, but not inoculated with OtV5. The dashed line represents the starting densities of the cultures at 10^5 cells ml^{-1}. There were three biological replicates for each populations. Boxes represent the interquartile range with the median indicated as the thick black line inside the box, and whiskers extend to the highest and lowest values within $1.5\times$ the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

that cell growth can increase in response to viruses in resistant populations. This occurs consistently in all replicates of a given population when it happens, but does not occur in all populations of a resistance type. We also see cases where lysis in some populations of S cells is incomplete, notably in the low light (NG′2) and low salt (NG′3) environments. Again, this does not occur in all populations, but it occurs reliably in replicates of the same population. While these effects of environment on lysis are not statistically significant because they do not occur over all populations within a resistance type, it could have evolutionary and ecological effects on the occasions when it does occur, which we discuss below.

Growth rate varied across environments regardless of resistance type

All populations were grown in a novel environment in the absence of OtV5 for 1 week, over which growth rate was measured. The response of *O. tauri* growth to the environment depended on resistance type (effect of environment × resistance type, $F_{8,114} = 4.45$, $p = 0.0001$). Additionally,

regardless of resistance type, population growth rates differed between environments (effect of environment on growth $F_{4,114} = 231.39$, $p < 0.0001$) (Fig. 2). Growth rates were higher in the control environment except for a single RP population, NG′10, which divided rapidly in the low salt environment (Fig. 2). Populations grown in the low phosphate environment all had reduced growth rates and showed less variation in growth than in all other environments.

The effect of resistance type on growth depends on environment

Resistance type alone did not significantly affect the growth rate of *O. tauri* ($F_{2,6} = 2.88$, $p = 0.1328$). This is because S and R cells had similar population growth rates in all environments (Fig. 2). In contrast, some populations of RP had different growth rates than both R and S cells. There was variation in growth rates between replicate populations of RP cells, with some populations consistently showing elevated growth rates. Two out of the three RP populations (NG′10 (shown by circle in Fig. 2) and NG′16 (shown by cross in Fig. 2) had higher growth rates than S and R cells
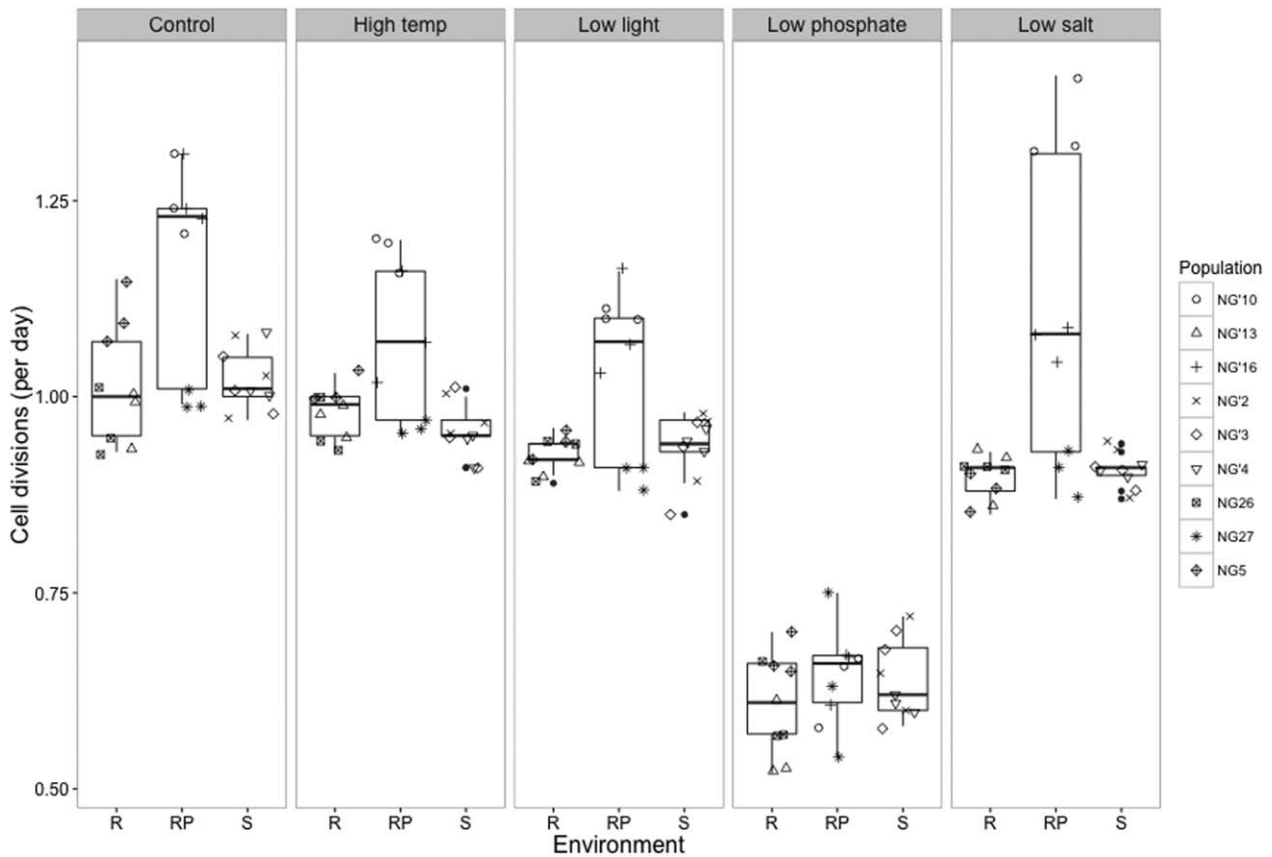
Fig. 2. Mean growth rates, measured as average number of cell divisions per day over 7 days, of susceptible (S), resistant (R) and resistant producer (RP) *O. tauri* cells grown in five environments in the absence of OtV5. There were three populations for each resistance type, with three biological replicates for each population. Boxes represent the interquartile range with the median indicated as the thick black line inside the box, and whiskers extend to the highest and lowest values within 1.5× the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

in four out of the five environments ($F_{3,5} = 17.19$, $p = 0.046$). The single exception was the low phosphate environment, where all resistance types had similar low growth rates. These data indicate that there is either no cost or an undetectable cost of resistance in terms of growth to either infection or lysis over a range of environments and that there can be a growth benefit of being resistant to lysis in some environments, as evidenced by the rapid growth of some RP populations. The low or absent cost of resistance is consistent with previous studies in single environments, which have reported costs of resistance detectable in competitions, but too low to be detectable by comparing growth rates (Thomas *et al.*, 2011).

Populations resistant to lysis can have a growth advantage in some environments

In order to assess whether the S, R and RP resistance types responded similarly to the different environments, environments were ranked from best to worst, based on population growth rates. All resistance types displayed

highest growth rates in the control environment (see Supporting Information Table S3). R cells had the same rank order of environments as the S cells. Since the growth rates of the RP cells were highly variable relative to the other resistance types, containing two populations that grew quickly, the RP populations were grouped into fast growing (NG'10 and NG'16) and normal growing (NG27). RP cells showed the same rank order of environments for both the fast and normal growing populations, except in low salt for the fast growing populations. This was due to one population (NG'10) displaying exceptionally high growth. Growth rate was the same in the low salt and low light environments for the normal growing RP population. Fast growing RP cells had higher cell growth in all environments except low phosphate.

To measure how sensitive growth rates were to environmental change, the slopes of the ranked environments were compared (Fig. 3). The two fast growing RP populations had a higher intercept (ANOVA effect of rank on growth, $F_{1,125} = 1112.56$, $p < 0.0001$), demonstrating the increase in growth rate compared to the other populations.
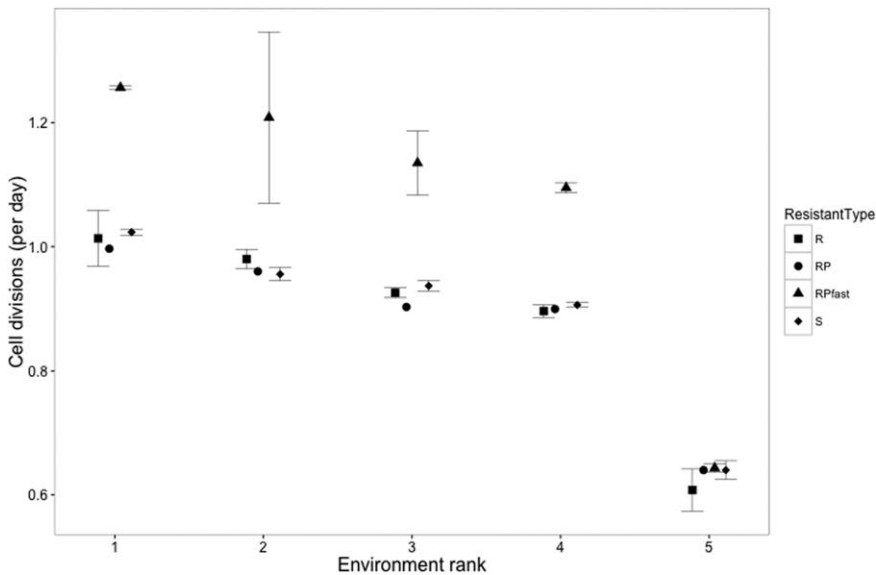
Fig. 3. Ranked environment by average cell divisions per day over 7 days (±SEM) for susceptible (S), resistant (R) and resistant producer (RP and RPfast) cells. Environments were ranked in order from best to worst for each resistance type based on growth rate in the absence of OtV5, where 1 is the environment with the highest growth rate. Fast and normal growing resistant producers have been plotted separately for visual purposes.

These data show that faster growing populations had a stronger preference for environments in which they can grow more quickly, however in the lowest ranking environment (which was low phosphate for all resistance types), these populations grew equally badly.

Size and chlorophyll content vary between cells with different resistance types in response to environment

After 1 week of growth in a novel environment without viruses, cell size and relative cell chlorophyll content were measured. Response of resistance type on cell size depended on environment (effect of environment × resistance type, $F_{8,114} = 5.48$, $p < 0.0001$). Regardless of resistance type, environment had a significant effect on cell size ($F_{4,114} = 77.93$, $p < 0.0001$). Cells were larger under low light ($t = 3.83$, $p = 0.0002$) and low phosphate conditions ($t = 7.49$, $p < 0.0001$), compared to the control environment (Fig. 4). No significant effect of resistance type was observed on cell size ($F_{2,6} = 0.01$, $p = 0.9945$). However, under low phosphate, there was a large variation in cell size between the fast and normal growing RP populations.

The two fast-growing RP populations had smaller cells than the normal growing RP population in the low phosphate environment. The RP population with normal growth had cells that were similar in size to the S populations (Fig. 4). To examine whether fast growing RP populations had different cell sizes than did populations with normal growth rates, *post hoc* models were used to analyse the two fast growing populations separately. Overall, no significant effect of resistance type was observed on cell size when normal and fast growing RP populations were analysed separately (ANOVA $F_{2,6} = 0.22$, $p = 0.8812$). Additionally, a model examining growth rate as a fixed effect was also performed. This showed a significant effect of growth rate ($F_{1,99} = 54.23$, $p < 0.0001$) and an interaction between resistance type and growth rate ($F_{2,99} = 4.64$, $p = 0.01$), although no effect of resistance type alone was detected ($F_{2,6} = 0.001$, $p = 0.99$). However, the statistical power in this data set, which contained only one population of normal growing RP cells and two populations of fast growing RP cells, was low, such that the chances of detecting an effect of resistance type on cell size is unlikely here even if one exists (power = 0.142).

The effect of resistance type on chlorophyll content per cell volume depended on environment (effect of environment × resistance type, $F_{8,114} = 10.68$, $p < 0.0001$). In addition, environment alone had a significant effect on relative chlorophyll per cell volume ($F_{4,114} = 120.45$, $p < 0.0001$), however resistance type alone did not ($F_{2,6} = 1.61$, $p = 0.2757$). Under low light, chlorophyll varied little between the three resistance types. In the other environments, S and R strategies usually displayed similar chlorophyll content levels with RP displaying lower chlorophyll levels in all environments except low phosphate.

By inspection, we see that the fast growing RP populations have less chlorophyll per cell volume than the normal growing RP population in all environments except low phosphate (Fig. 5). We used a *post hoc* model with growth rate as a fixed effect to investigate whether the fast growing RP populations also had different chlorophyll contents. Growth rate had a significant effect on chlorophyll content ($F_{1,99} = 57.86$, $p < 0.0001$), with fast growing RP populations having lower chlorophyll content, and the effect of growth rate was dependent on environment ($F_{4,99} = 3.85$, $p = 0.01$) and resistance type ($F_{2,99} = 6.27$, $p = 0.003$). Furthermore, when growth rate was considered in the
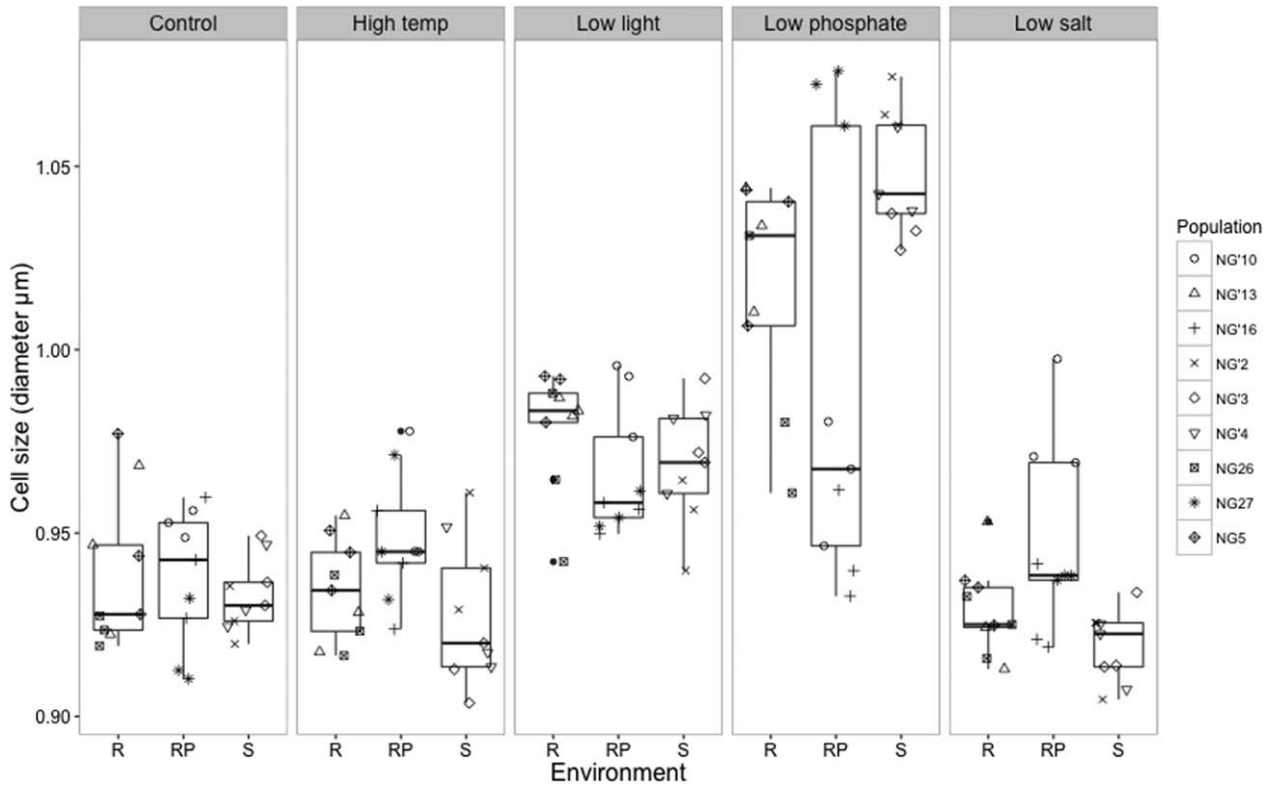
Fig. 4. Mean cell size for susceptible (S), resistant (R) and resistant producer (RP) cells after seven days of growth in the absence of viruses in five environments. There were three populations for each resistance type, with three biological replicates for each populations. Boxes represent the interquartile range with the median indicated as the thick black line inside the box, and whiskers extend to the highest and lowest values within 1.5× the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

model, resistance type alone had a significant effect on chlorophyll content ($F_{2,6} = 5.49$, $p = 0.04$), suggesting that the growth rate of the fast growing RP populations reduced chlorophyll content.

Discussion

Effect of environment on host resistance

We observed no differences in susceptibility of any of the populations to OtV5 over the environments tested. While the ability of the virus to lyse host cells did not depend on the environment, R and RP cells had different growth responses to viral exposure. There were two cases in which a resistant population repeatedly had a higher cell density after exposure to OtV5 than its paired control culture that was not inoculated. We speculate that this may be a response to the virus, which causes the phytoplankton cells to divide more rapidly. This would be advantageous if, for example, a population that was made up of mixed susceptible and resistant cells were exposed to virus—any resistant cell lineages that could increase their growth rate would then take over the population by overgrowing any remaining resistant cells whose growth rate was unaffected by exposure to virus.

We did not detect a growth cost of resistance when R and RP populations were grown in the absence or presence of OtV5 after exposure to a novel environment. We expect to see a trade-off for being resistant to viral infection, because if there were no cost there should be a strong selection pressure for all cells to become resistant, yet we still find susceptible populations both in the laboratory and in the ocean (Thomas *et al.*, 2011; Clerissi *et al.*, 2012). Previous work shows that that susceptible cells can have a competitive advantage against resistant cells (Lenski, 1988). Additionally, we speculate that resistance to one virus strain could make these cells susceptible to other OtVs. Clerissi *et al.* (2012) showed that OtVs are mainly intraspecies-specific and that hosts that are the most resistant to infection can often be infected by more generalist viruses. This specificity could be caused by proteins involved in adaptive behaviour (Clerissi *et al.*, 2012). Thus, we suggest that in addition to the abiotic environment, biotic environment could play a large role in *O. tauri* resistance strategy.

Since viruses are responsible for a large proportion of microbial death, there is strong selection on hosts for resistance or tolerance to viral infection. There are several suggestions to explain the paradox of how susceptible
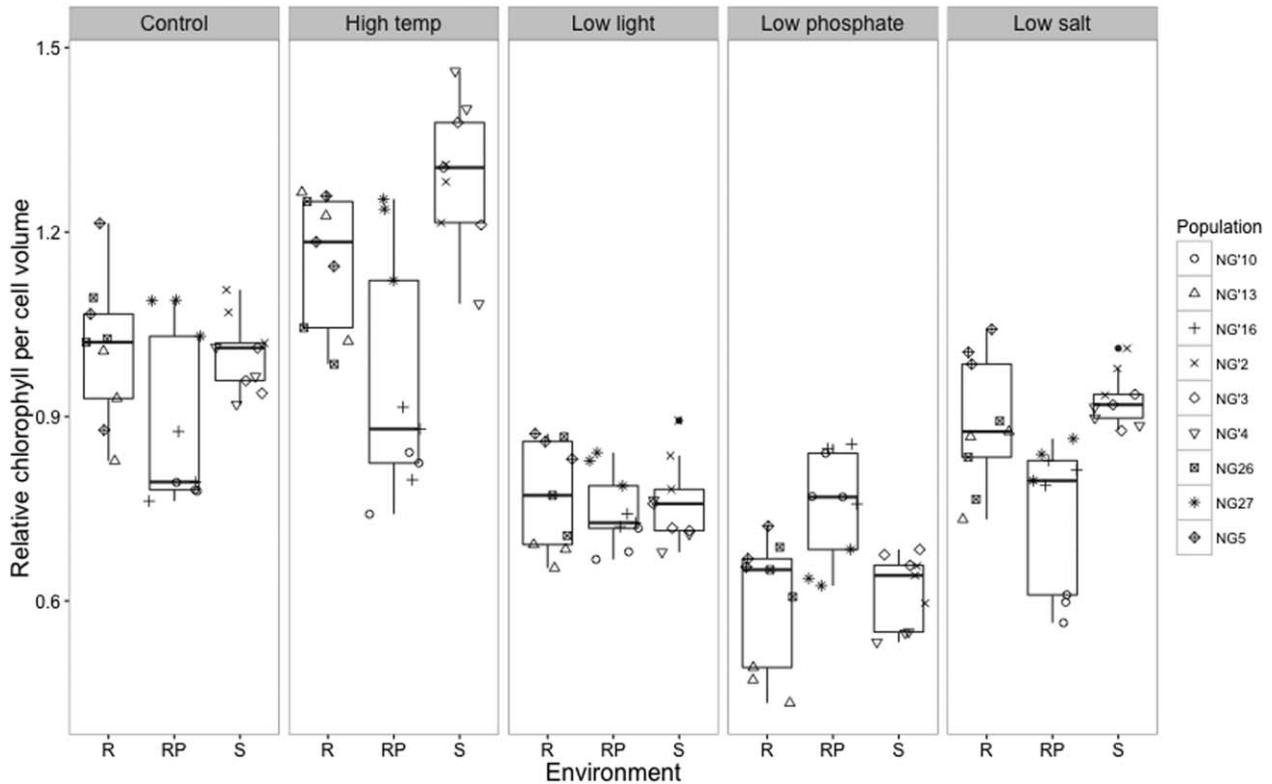
Fig. 5. Mean relative chlorophyll to cell size for susceptible (S), resistant (R) and resistant producer (RP) cells after seven days of growth in the absence of viruses in five environments. There were three populations for each resistance type, with three biological replicates for each population. Boxes represent the interquartile range with the median indicated as the thick black line inside the box, and whiskers extend to the highest and lowest values within 1.5× the inter-quartile range from the edge of the box. Outlier data beyond the end of the whiskers are plotted as points.

algal cells and their viruses are able to co-exist in marine environments without extinction of the host. One theory as to how viruses and their hosts are able to coexist is that there must be a cost to being resistant to infection. This is often expected to be a reduction in growth (Weinbauer, 2004), as has been observed in *Synechococcus*, in which there was a 20% reduction in fitness compared to the ancestor in resistant strains (Lennon *et al.*, 2007). Thus in the absence of viruses, resistant cells often have a lower fitness. This could lead to decreased numbers in the absence of viruses. An evolutionary 'arms race' may occur when viruses and their hosts adapt reciprocally to overcome resistance and infection, respectively. We find little evidence for a cost of resistance in our study, but this may be because the laboratory environments used are missing a key aspect of the natural environment that, if present, results in a cost of resistance in *O.tauri*. Alternatively, although deviating from the standard control environment, none of the environments in this study were severely stressful, with even the low phosphate environment allowing reasonable growth. Thus, it is possible that we did not detect a growth cost because the changes to the environments used were relatively modest.

Various strategies for virus resistance have been reported in algae, including activation of programmed cell death (Bidle *et al.*, 2007), absence of metacaspase (caspase orthologues) protein expression (Bidle *et al.*, 2007), stage of the life cycle (Frada *et al.*, 2008), changes to cell surface receptor proteins (Tarutani *et al.*, 2006), colony formation (Brussaard *et al.*, 2007) and genetic mutations (Stoddard *et al.*, 2007). However, it is still unknown how *O. tauri* cells acquire their two resistance strategies. We found that short-term exposure to novel environments does not affect resistance type and we did not observe any cost of resistance leading to cells losing their resistance to OtV5.

Effect of resistance type on population growth and other phenotypic traits

We found that after 1 week in a novel environment, growth rate of *O. tauri*, as measured by the average number of cell divisions per day over seven days, varied across environments for all resistance types. RP populations had the fastest average rates of cell division in most environments. All resistance types showed the same environmental preferences, with average cell division rates highest in the

control environment. The only exception was one RP population that divided rapidly in the low salt environment. The lowest growth rates were observed in the low phosphate environment, which was expected since these cells were deprived of a key nutrient.

Two of the three RP populations divided more rapidly than all of the S and R populations. These populations were fast growing in many environments, including the control environment, suggesting that the rapid growth is a general character of these two RP populations, rather than a response to stress or novelty. This faster growth rate in RP populations relative to S and R populations is in contrast with previous studies on *O. tauri*. Thomas *et al.* (2011) detected no difference in growth rate between S, R and RP cells, although competition experiments revealed a small reduction of fitness in RP compared to R, and R compared to S. Our results suggest that the opposite can be true. Similarly to Thomas *et al.* (2011), we did not observe a fitness cost in terms of growth rate for the remaining populations since S, R and normal growing RP populations had similar growth rates across environments. This was expected, at least in the control environment, where previous studies have only been able to detect a minimal cost of resistance by using direct competitions. Surprisingly, the two fast growing RP populations could not be detected as having more rapid growth under low phosphate, however these populations responded differently in their size and chlorophyll contents.

Reduced growth rate is often observed as a cost of resistance in microbes and has been measured in several species (Lennon *et al.*, 2007; Haaber and Middelboe, 2009). Ecologically, a cost of resistance is part of 'kill the winner' dynamics, where, it is hypothesized that viruses kill the faster growing (susceptible) cells, and thus provide an opportunity for slower growing (resistant) cells (Mojica and Brussaard, 2014). This role for viruses requires that there be a cost of resistance. However, here we did not detect a cost of resistance in terms of growth rate, since there was no environment in which resistant cell types grew at slower rates than S cells. In fact, we observed the opposite in two out of the three RP populations, where resistant cells grew faster than the S populations across all environments except low phosphate. In cases where resistant cells (R or RP populations) did not divide faster than susceptible ones, they divided at the same rate. Taken together, this suggests that the cost of resistance to OtVs is likely to be small or absent, and may not play into kill the winner dynamics. This opens the question of how the appearance of resistance to OtVs affects both host and viral ecology.

Environment affected cell size, whereas generally, resistance type did not. However, under low phosphate, the two fast growing RP populations were smaller than the normal growing RP populations, suggesting that under nutrient limitation these cells were able to divide at a smaller cell size. Smaller phytoplankton cell size is often selected for in nutrient limited environments since smaller cells have a larger surface area to volume ratio and a thinner diffusion boundary layer, thus facilitating nutrient uptake (Finkel *et al.*, 2010; Peter and Sommer, 2015). Although fast growing RP populations in this selection environment were smaller than the normal growing RP population, their cell size was not different from the fast growing RP populations in the other environments. The control was the only environment in which fast growing RP populations were larger than the normal growing populations, indicating that there may be a (direct or indirect) fitness benefit associated with the increased size of the RP type under control conditions.

In contrast to previous studies, all populations in the low phosphate environment, except fast growing RP, increased in cell size. Cell division of larger phytoplankton cells requires greater nutrient concentrations, which can decrease the division rate. Since cells in the low phosphate environment had a reduced growth rate in terms of cell divisions, this could have resulted in cells that reached a larger volume even though the environment was phosphate-poor. It has previously been suggested that increasing algal cell size, and thus the volume to surface area ratio, can facilitate reduced phosphorus uptake under phosphate-limited conditions, and that this adaptation response may be more favourable than decreasing cell size (Šupraha *et al.*, 2015). A common response of coccolithophores to phosphate limitation is reduced growth rate and increased cell size (Šupraha *et al.*, 2015).

Smaller phytoplankton cells have often been observed growing at higher temperatures in natural environments, which is thought to arise from the temperature-size rule (e.g., Atkinson *et al.*, 2003; Morán *et al.*, 2010). These studies used large temperature ranges, but there was no effect of the modest increase in temperature on cell size in this study. Smaller cells have also been reported to cope better with both light limitation and light saturation compared to larger cells due to a reduction in internal shading (Geider *et al.*, 1986; Raven, 1998; Finkel *et al.*, 2010). We found no significant difference in cell size under low light, although there was a non-significant trend for cells to be slightly larger in low than under control light.

Environment was found to have a significant effect on chlorophyll content per cell volume, whereas resistance type alone had no effect. We observed lower chlorophyll per cell volume in all environments compared to the control except high temperature. Although resistance type alone did not have an effect, growth rate had a significant effect on chlorophyll content per cell volume when included in the model and normal growing S, R and RP cells in the control and high temperature environments had the highest chlorophyll levels across all environments. In contrast, fast growing RP populations showed no significant difference in chlorophyll content per cell volume across all five

environments. All populations had their lowest growth rates in the low phosphate environment and cells in this environment had the lowest chlorophyll content, except for fast growing RP populations. Fast growing RP populations had lower chlorophyll content than the normal growing RP population in all environments except for low phosphate.

One experiment using cultures of different phytoplankton groups found that chlorophyll content was lower during both nitrogen and phosphorus depletion (Riemann *et al.*, 1989). Additionally, phytoplankton cells grown under low nutrients have been observed to decrease their photosynthesis rates (Litchman *et al.*, 2003; Spilling *et al.*, 2015). This may be due to the cells allocating resources to synthesizing chloroplasts under nutrient limitation. In our study the control environment was the preferred one, and it is possible that cells were unable to synthesise large quantities of chlorophyll in the other (less permissive) environments since their energy was allocated to growth. It is possible that under elevated temperature, the metabolism of *O. tauri* was increased, leading the cells to synthesise more chlorophyll. Temperature did not affect chlorophyll a content in diatoms (Sigaud and Aidar, 1993). Salinity appears to affect different phytoplankton species differently, with some species showing no change in chlorophyll content across a range of salinities, and others having higher chlorophyll contents at the optimum salinity for growth (McLachlan, 1961; Sigaud and Aidar, 1993).

Concluding remarks

Resistance of microbes to virus infection often comes at a cost, with one common observation being a reduction in growth compared to susceptible cells in the population. In this study, our aim was to measure resistance to viruses in *O. tauri* across different environments and to determine whether the magnitude of a cost of resistance depends on environmental context. We did not observe a cost of resistance as measured by cell divisions, cell size or chlorophyll content in the present study. Growth rates of *O. tauri* were reduced when grown in low phosphate, however this did not affect the ability of OtV5 to lyse susceptible cells in this environment. Additionally, although growth rates were lower than the controls in high temperature, low light and low salinity, OtV5 still caused cell lysis of susceptible cells. Indeed, some populations that were tolerant to infection (RP populations) had evolved high growth rates, and some RP populations also increased their growth rates after exposure to viruses. Both observations suggest that resistance strategy could have interesting ecological consequences by changing the relative fitness of different populations.

Table 1. A comparison of the control environment and the environment treatments that were used for each environmental condition in this study.

Environment	Control	Treatment
Phosphate (μM)	10	5
Salinity (ppt)	30	25
Light (μmol m^{-2} s^{-1})	85	60
Temperature ($^{\circ}$C)	18	20

Experimental procedures

Susceptible and resistant populations used in this experiment

O. tauri populations were obtained from N. Grimsley, Observatoire Océanologique, Banyuls-sur-Mer. Three susceptible populations (NG′2, NG′3 and NG′4), three resistant producer populations (NG′10, NG′16 and NG27) and three resistant populations (NG5, NG′13 and NG26) were used in this study. We used three biological replicates for each population in each environment. All populations were derived from a single clone of *O. tauri* (RCC 4221) and therefore had the same starting genotype (see Thomas *et al.*, 2011). All populations have since been maintained separately. All RP populations were tested for viral production prior to the start of the experiment (See Supporting Information Fig. S1).

Culturing conditions

Populations were grown in batch culture. Culture medium was prepared using 0.22 μm filtered Instant Ocean artificial seawater (salinity 30 ppt) aerated with 400 ppm CO_2 and supplemented with Keller and f/2 vitamins. Control cultures were maintained in a 14:10 light:dark cycle at 85 μmol photon m^{-2} s^{-1} and at a constant temperature of 18°C (Table 1).

For the selection experiments, *O. tauri* populations were grown without exposure to viruses in the control environment and four selection environments. The selection regimes used were high temperature, low light, low phosphate and low salinity (Table 1). Cultures were acclimated in each selection environment for 1 week, followed by 1 week of growth in each environment.

For the low phosphate environment, phosphate was reduced by preparing Keller media with only half the amount of β-glycerophosphate that would normally be used. Although the phosphate concentration in the low phosphate environment is not low compared to natural seawater [0.01–2.99 μmol l^{-1} in the Leucate lagoon where *O. tauri* and OtV5 inhabit (Clerissi *et al.*, 2014)], it is low compared to the control media in which the populations had been maintained prior to the experiment. For culture medium with a lower salinity than the control, Instant Ocean was added to reach a salinity of 25 ppt. For the low light condition, culture flasks were wrapped in 0.15 neutral density foil to give a light intensity of 1000 lux. Cultures in the high temperature condition were maintained on a heat mat (Exo Terra Heat Wave substrate heat mat) set at 20°C.

The effect of viral exposure on cell division rates

Following 1 week of growth in the selection environment, each sample was inoculated with a fresh suspension of OtV5 particles to test whether it was susceptible or resistant to the virus. Samples were tested by inoculating 1 ml cell culture at a density of 10^5 with 10 µl OtV5 in 48-well plates with three replicates for each sample. Controls that were not inoculated with viruses were used as a control for cell growth. Cell density was measured using a FACSCanto flow cytometer 3 days after inoculation.

Population growth of susceptible and resistant populations across different environments

Following the acclimation period, average cell densities per day of all cultures were measured over 1 week of growth in each environment. Cells were counted using a BD FACS-Canto II (BD Biosciences) flow cytometer before the first transfer and after seven days of growth. Each population was counted in triplicate. The cell counts were converted to cells per millilitre and the number of divisions per day was calculated using Eq. (1).

$$\mu\ (d^{-1}) = \frac{\log_2\left(\frac{N_t}{N_0}\right)}{t - t_0}, \tag{1}$$

where N_t and N_0 are the cell densities (cells ml^{-1}) at times t_1 and t_0 (days). This measures the average number of cell divisions per ancestor over a single growth cycle and allows a comparison of offspring production between environments (Brennan and Collins, 2015). This is useful if different environments produce different growth curves since populations with different growth strategies can be compared. This calculation is also not sensitive to small differences in N_0, which is important if the population size reached during the acclimation period differs between environments or resistance types.

Cell size and chlorophyll content of populations with different resistance types across environments

Cell size and relative chlorophyll content per cell volume were determined using a FACSCanto flow cytometer. Cell size was inferred from FSC (forward scatter), which was calibrated using beads of known sizes (1, 3 and 6.6 µm). Chlorophyll fluorescence was inferred by measuring PerCP-Cy5.5 emission with excitation at 488 nm. Relative chlorophyll was analysed by taking the average chlorophyll fluorescence for all susceptible populations in the control environment and setting this to a value of 1, with chlorophyll measurements of all populations relative to this value.

Statistical analysis

Data were analysed with linear mixed effects models using the statistical package nlme in R (version 3.2.0) to identify differences in growth rates between the different environments after 1 week of growth and after virus inoculation. Environment and resistance type were fixed effects when analyzing growth under different environments, and environment, resistance type and treatment were fixed effects when analyzing virus inoculation under different environments. Population was a random effect in both models.

Post hoc mixed effects models were used to examine whether growth rate had an effect on cell size and chlorophyll content in cells. Environment, resistance type and growth rate (cells divisions per day) were set as fixed effects with populations as the only random effect.

Data

All data and R scripts are available from the Dryad Digital Repository doi:10.5061/dryad.344qn.

References

Atkinson, D., Ciotti, B.J., and Montagnes, D.J.S. (2003) Protists decrease in size linearly with temperature: ca. 2.5% degrees C(-1). *Proc Biol Sci* **270:** 2605–2611.

Avrani, S., and Lindell, D. (2015) Convergent evolution toward an improved growth rate and a reduced resistance range in Prochlorococcus strains resistant to phage. *Proc Natl Acad Sci U S A* **112:** E2191–E2200.

Bellec, L., Grimsley, N., Derelle, E., Moreau, H., and Desdevises, Y. (2010) Abundance, spatial distribution and genetic diversity of *Ostreococcus tauri* viruses in two different environments. *Environ Microbiol Rep* **2:** 313–321.

Bidle, K.D., Haramaty, L., Barcelos E Ramos, J., and Falkowski, P. (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi. Proc Natl Acad Sci U S A* **104:** 6049–6054.

Bohannan, B.J.M., Kerr, B., Jessup, C.M., Hughes, J.B., and Sandvik, G. (2002) Trade-offs and coexistence in microbial microcosms. *Antonie Van Leeuwenhoek*, **81:** 107–115.

Børsheim, K.Y. (1993) Native marine bacteriophages. *FEMS Microbiol Lett* **102:** 141–159.

Bratbak, G., Egge, J.K., and Heldal, M. (1993) Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms. *Mar Ecol Prog Ser* **93:** 39–48.

Bratbak, G., Jacobsen, A., Heldal, M., Nagasaki, K., and Thingstad, F. (1998) Virus production in *Phaeocystis pouchetii* and its relation to host cell growth and nutrition. *Aquat Microb Ecol* **16:** 1–9.

Brennan, G., and Collins, S. (2015) Growth responses of a green alga to multiple environmental drivers. *Nat Clim Change* **5:** 892–897.

Brussaard, C.P.D., Bratbak, G., Baudoux, A.C., and Ruardij, P. (2007) Phaeocystis and its interaction with viruses. *Biogeochemistry* **83:** 201–215.

Bruussard, C.P. (2004) Viral control of phytoplankton populations—A review. *J Eukaryot Microbiol* **51:** 125–138.

Chen, S., Gao, K., and Beardall, J. (2014) Viral attack exacerbates the susceptibility of a bloom-forming alga to ocean acidification. *Global Change Biol* 21, 629–636.

Clerissi, C., Desdevises, Y., and Grimsley, N. (2012) Prasinoviruses of the marine green alga *Ostreococcus tauri* are mainly species specific. *J Virol* **86:** 4611–4619.

Clerissi, C., Grimsley, N., Subirana, L., Maria, E., Oriol, L., Ogata, H., *et al.* (2014) Prasinovirus distribution in the Northwest Mediterranean Sea is affected by the environment and particularly by phosphate availability. *Virology* **466–467:** 146–157.

Danovaro, R., Corinaldesi, C., Dell'anno, A., Fuhrman, J. A., Middelburg, J.J., Noble, R.T., and Suttle, C.A. (2011) Marine viruses and global climate change. *FEMS Microbiol. Rev* **35:** 993–1034.

Derelle, E., Ferraz, C., Escande, M.L., Eychenié, S., Cooke, R., Piganeau, G., *et al.* (2008) Life-cycle and genome of OtV5, a large DNA virus of the pelagic marine unicellular green alga *Ostreococcus tauri*. *PLoS One* 3: e2250.

Finkel, Z.V., Beardall, J., Flynn, K.J., Quigg, A., Rees, T.A.V., and Raven, J.A. (2010) Phytoplankton in a changing world: cell size and elemental stoichiometry. *J Plankton Res* **32:** 119–137.

Frada, M., Probert, I., Allen, M.J., Wilson, W.H., and de Vargas, C. (2008) The 'Cheshire Cat' escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection. *Proc Natl Acad Sci U S A* **105:** 15944–15949.

Frickel, J., Sieber, M., and Becks, L. (2016) Eco-evolutionary dynamics in a coevolving host-virus system. *Ecol Lett* **19:** 450–459.

Geider, R., Platt, T., and Raven, J. (1986) Size dependence of growth and photosynthesis in diatoms: a synthesis. *Mar Ecol Ser* **30:** 93–104.

Haaber, J., and Middelboe, M. (2009) Viral lysis of *Phaeocystis pouchetii*: implications for algal population dynamics and heterotrophic C, N and P cycling. *Isme J* 3: 430–441.

Jacquet, S., and Bratbak, G. (2003) Effects of ultraviolet radiation on marine virus-phytoplankton interactions. *FEMS Microbiol Ecol* **44:** 279–289.

Larsen, J.B., Larsen, A., Thyrhaug, R., Bratbak, G., and Sandaa, R.-A. (2007) Marine viral populations detected during a nutrient induced phytoplankton bloom at elevated pCO_2 levels. *Biogeosci Discuss* 4: 3961–3985.

Lennon, J.T., Khatana, S.A.M., Marston, M.F., and Martiny, J.B.H. (2007) Is there a cost of virus resistance in marine cyanobacteria?. *Isme J* 1: 300–312.

Lenski, R.E. (1988) Experimental studies of pleiotropy and epistasis in Escherichia coli. I. Variation in competitive fitness among mutants resistant to virus T4. *Evolution (N. Y)* **42:** 425–432.

León, M., and Bastías, R. (2015) Virulence reduction in bacteriophage resistant bacteria. *Front Microbiol* 6: 1–7.

Litchman, E., Steiner, D., and Bossard, P. (2003) Photosynthetic and growth responses of three freshwater algae to phosphorus limitation and daylength. *Freshw Biol* **48:** 2141–2148.

Maat, D., Crawford, K., Timmermans, K., and Brussard, C. (2014) Elevated CO_2 and phosphate limitation favor *Micromonas pusilla* through stimulated growth and reduced viral impact. *Appl Environ Microbiol 80*: 3119–3127.

Marston, M.F., Pierciey, F.J., Shepard, A., Gearin, G., Qi, J., Yandava, C., *et al.* (2012) Rapid diversification of coevolving

marine Synechococcus and a virus. *Proc Natl Acad Sci U S A* 109: 4544–4549.

McLachlan, J. (1961) The effect of salinity on growth and chlorophyll content in representative classes of unicellular marine algae. *Can J Microbiol* 7: 399–406.

Mojica, K.D.A., and Brussaard, C.P.D. (2014) Factors affecting virus dynamics and microbial host-virus interactions in marine environments. *FEMS Microbiol Ecol* **89:** 495–515.

Morán, X.A.G., López-Urrutia, Á., Calvo-Díaz, A., and Li, W.K.W. (2010) Increasing importance of small phytoplankton in a warmer ocean. *Global Change Biol* **16:** 1137–1144.

Nagasaki, K., and Yamaguchi, M. (1998) Effect of temperature on the algicidal activity and the stability of HaV (*Heterosigma akashiwo* virus). *Aquat. Microb. Ecol* **15:** 211–216.

Peter, K.H., and Sommer, U. (2015) Interactive effect of warming, nitrogen and phosphorus limitation on phytoplankton cell size. *Ecol Evol* **5:** 1011–1024.

Raven, J.A. (1998) The twelfth Tansley Lecture. Small is beautiful: The picophytoplankton. *Funct Ecol* **12:** 503–513.

Riemann, B., Simonsen, P., and Stensgaard, L. (1989) The carbon and chlorophyll content of phytoplankton from various nutrient regimes. *J Plankton Res* **11:** 1037–1045.

Seed, K.D., Faruque, S.M., Mekalanos, J.J., Calderwood, S.B., Qadri, F., and Camilli, A. (2012) Phase variable O antigen biosynthetic genes control expression of the major protective antigen and bacteriophage receptor in *Vibrio cholerae* O1. *PLoS Pathog* 8: e1002917.

Sigaud, T.C.S., and Aidar, E. (1993) Salinity and temperature effects on the growth and chlorophyll-a content of some planktonic aigae. *Bol Do Inst Ocean* **41:** 95–103.

Spilling, K., Ylöstalo, P., Simis, S., and Seppälä, J. (2015) Interaction effects of light, temperature and nutrient limitations (N, P and Si) on growth, stoichiometry and photosynthetic parameters of the cold-water diatom *Chaetoceros wighamii*. *PLoS One* **10:** 1–18.

Stoddard, L.I., Martiny, J.B.H., and Marston, M.F. (2007) Selection and characterization of cyanophage resistance in marine Synechococcus strains. *Appl Environ Microbiol* **73:** 5516–5522.

Šupraha, L., Gerecht, A.C., Probert, I., and Henderiks, J. (2015) Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation. *Sci Rep* **5:** 16499.

Tarutani, K., Nagasaki, K., and Yamaguchi, M. (2006) Virus adsorption process determines virus susceptibility in *Heterosigma akashiwo* (Raphidophyceae). *Aquat Microb Ecol* **42:** 209–213.

Thomas, R., Grimsley, N., Escande, M.L., Subirana, L., Derelle, E., and Moreau, H. (2011) Acquisition and maintenance of resistance to viruses in eukaryotic phytoplankton populations. *Environ Microbiol* **13:** 1412–1420.

Weinbauer, M.G. (2004) Ecology of prokaryotic viruses. *FEMS Microbiol Rev* **28:** 127–181.

Wells, L.E., and Deming, J.W. (2006) Effects of temperature, salinity and clay particles on inactivation and decay of cold-active marine Bacteriophage 9A. *Aquat Microb Ecol* **45:** 31–39.

Weynberg, K.D., Allen, M.J., Ashelford, K., Scanlan, D.J., and Wilson, W.H. (2009) From small hosts come big viruses: the complete genome of a second *Ostreococcus tauri* virus, OtV-1. *Environ Microbiol* **11:** 2821–2839.

Weynberg, K.D., Allen, M.J., Gilg, I.C., Scanlan, D.J., and Wilson, W.H. (2011) Genome sequence of *Ostreococcus*

tauri virus OtV-2 throws light on the role of picoeukaryote niche separation in the ocean. *J Virol* **85:** 4520–4529.

Wilhelm, S.W., and Suttle, C.A. (1999) Viruses and nutrient cycles in the sea. *Bioscience* **49:** 781–788.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Mean cell densities ml^{-1} ($\pm$SEM) of *O. tauri* strain RCC4221 three days after inoculation with supernatant from Resistant Producing populations (NG'10, NG'16 and NG27). To ensure that the RP populations being used in this experiment were producing infectious viruses and releasing them to their external surroundings, we used the supernatant of these strains to infect susceptible *O. tauri* cells. Populations NG'10, NG'16 and NG'27 were aliquoted into 2 ml Eppendorf tubes and centrifuged at 8000 $\times$ g for 15 min. Next, 400 μl of supernatant was carefully removed without drawing up any cells from the pellet at the bottom of the tube, and used to inoculate 1 ml of susceptible *O. tauri* strain RCC4221. Eight replicates were performed. A positive control was performed using known OtV5, and a negative control was performed by adding Keller media. Controls were performed in quadruplicate. Cells were left to grow for 3 days after which their densities were measured using a FACSCanto flow cytometer. We observed cell lysis resulting from inoculation with supernatant from all three RP populations, showing that there was active virus in the media taken from these cultures.

Table S1. ANOVA results of a linear mixed effects model to analyse interaction effects of environment, resistance type and treatment (with or without OtV5 inoculation) on *O. tauri* cell density. Population was a random effect.

Table S2. ANOVA results of a linear mixed effects model to analyse interaction effects of environment, resistance type, treatment (with or without OtV5 inoculation) and growth rate (fast RP or normal) on *O. tauri* growth rate, as measured by cell divisions per day. Population was set as a random effect in all models.

Table S3. Ranked environments by fitness as measured by cell divisions per day for each resistance type. Environments were ranked in order from best to worst, where 1 is the environment with the highest growth rate. Fast and normal growing resistant producers were ranked separately to compare slopes.

Virus Resistance Is Not Costly in a Marine Alga Evolving under Multiple Environmental Stressors

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Abstract: Viruses are important evolutionary drivers of host ecology and evolution. The marine picoplankton *Ostreococcus tauri* has three known resistance types that arise in response to infection with the Phycodnavirus OtV5: susceptible cells (S) that lyse following viral entry and replication; resistant cells (R) that are refractory to viral entry; and resistant producers (RP) that do not all lyse but maintain some viruses within the population. To test for evolutionary costs of maintaining antiviral resistance, we examined whether *O. tauri* populations composed of each resistance type differed in their evolutionary responses to several environmental drivers (lower light, lower salt, lower phosphate and a changing environment) in the absence of viruses for approximately 200 generations. We did not detect a cost of resistance as measured by life-history traits (population growth rate, cell size and cell chlorophyll content) and competitive ability. Specifically, all R and RP populations remained resistant to OtV5 lysis for the entire 200-generation experiment, whereas lysis occurred in all S populations, suggesting that resistance is not costly to maintain even when direct selection for resistance was removed, or that there could be a genetic constraint preventing return to a susceptible resistance type. Following evolution, all S population densities dropped when inoculated with OtV5, but not to zero, indicating that lysis was incomplete, and that some cells may have gained a resistance mutation over the evolution experiment. These findings suggest that maintaining resistance in the absence of viruses was not costly.

Keywords: evolution; trade-off; cost of resistance; Phycodnavirus; Prasinovirus; environmental change; virus-host interactions; marine viral ecology; *Ostreococcus tauri*

1. Introduction

Viruses are the most abundant biological entities in the oceans, with an estimated 10^{30} particles globally [1]. Viruses play a key role in marine food webs, partially because viral infection of unicellular organisms often results in cell lysis, where the infected cell bursts to release the new viruses; products of lysis feed back into the microbial loop and provide organic matter to organisms at the base of the food web daily [2]. In addition to being a large cause of mortality to their hosts, viruses can exert strong selection on host immune defense, leading to the evolution of host resistance mechanisms. Strong immune defenses, in turn, impose strong selection on viruses to evade these resistance responses leading to an ongoing co-evolutionary process between hosts and viruses [3]. Experimental evidence of host-virus coevolution has come mainly from bacteria-phage systems [3,4]. Viruses evolve rapidly due to their small size and high mutation rates [5] which can strongly influence the evolution of their hosts. However, in addition to infection, hosts are also subject to other selection pressures, such as

severe or stressful environmental changes. In the case of marine hosts, they will be subject to natural selection both from their viruses, and from, for example, the changes in nutrients, temperature and light associated with global change in the oceans [6], which opens up the possibility that the genetic and physiological changes associated with resistance may affect host evolution in response to challenges other than the virus itself. This in turn has the potential to affect how primary productivity at the base of marine food webs evolves in response to global change. Studies have examined environmental effects on interactions between microalgae and their viruses under a range of conditions including changes in temperature [7,8], nutrients [9–13], UV radiation [14], light intensity [11,15,16], and CO_2 levels [13,17,18]. Environmental change can have direct effects on marine viruses, for example by damaging and/or deactivating the particles through UV exposure or extreme temperatures [8,14]. However, viral abundance is thought to be mainly dependent on host availability and, therefore, the effects of environmental change on viruses are expected to be mainly indirect (e.g., [19]). Here we focus on host evolution rather than viral selection.

Hosts are capable of evolving resistance to their viruses, though resistance often entails a fitness cost, which can vary in form and magnitude [20]. Costs of resistance that have been reported in microorganisms include reduced competitive ability [20,21], reduced growth rate [22,23], reduced original function of a receptor protein [24,25], and increased susceptibility to other viruses [26–28]. If the cost of resistance is substantial and related to growth or competitive ability, resistance might be lost when the selection pressure for it is removed (i.e., when viruses are absent) [29]. For example, under conditions where viruses are present and able to interact with their host cells, resistant hosts should have a selective advantage over susceptible hosts by avoiding lysis. However, in the absence of viruses, the selection pressure for resistance is removed and costs of resistance, if present and substantial, should reduce host fitness, so that there is an advantage to losing resistance. Most studies have focused on costs of resistance in bacteria (e.g., [22,28,30,31]), however data for eukaryotic microalgae are lacking, which limits our ability to translate the literature on host-virus interactions to primary producers in the oceans. Because marine algae are the dominant primary producers in oceans [32], changes in the abundance, distribution and composition of microalgal assemblages in response to climate change are likely to have important implications for marine communities.

The marine picoeukaryote *Ostreococcus tauri* and its viruses, *Ostreococcus tauri* viruses (OtVs), are abundant in Mediterranean lagoons [33]. OtVs are lytic viruses belonging to the family Phycodnaviridae that cause susceptible (S) host *O. tauri* cells to burst following infection [34]. However, two resistant host types have been observed [35,36]. In the first type, viruses can attach to the resistant (R) host cells but are unable to replicate and cause lysis. In the second type, resistant producer (RP) populations consist mainly of resistant cells with a minority of susceptible cells (<0.5%) that maintains a population of viruses. These two resistance mechanisms have been observed repeatedly and remain resistant to lysis over many generation of sub-culturing [35,36]. Previous work found that there was no difference in growth rates between the three resistance types when they were maintained separately under standard laboratory culturing conditions, although long term competitions indicated a cost of resistance with susceptible cells outcompeting resistant cells and resistant cells outcompeting resistant producers after 100 and 200 days, respectively [35].

This study examined whether a cost of resistance could be detected in *O. tauri* in terms of the ability to adapt to different environmental conditions, and whether the evolutionary responses to environmental change were affected by resistance type. Populations of S, R and RP *O. tauri* were evolved under different environmental conditions in the absence of viruses for 200 generations to answer whether resistance type was maintained and how resistance type affected evolutionary responses, even in the absence of coevolutionary dynamics imposed by the presence of viruses. We found that all R and RP populations remained resistant to OtV5 inoculation across all environments, whereas S populations had a lower proportion of cell lysis at the end than at the start of the evolution experiment. Additionally, resistance type affected cell division rates, size and chlorophyll content, whereas selection environment affected cell division rates and competitive ability.

2. Materials and Methods

2.1. Susceptible and Resistant Lines

O. tauri lines were obtained from N. Grimsley, Observatoire Océanologique, Banyuls-sur-Mer, France. Three susceptible lines (NG'2, NG'3 and NG'4), three resistant lines (NG5, NG'13 and NG26) and three resistant producer lines (NG'10, NG'16 and NG27) were used. All lines were derived from a single clone of *O. tauri* (RCC4221) and therefore had the same starting genotype.

2.2. Culturing Conditions

For each of the nine lines described above, three biological replicates were evolved per environment (27 independent populations in total per environment). We refer to each independent replicate as a population. Populations were grown in batch culture. Culture medium was prepared using 0.22 μm filtered Instant Ocean artificial seawater (salinity 30 ppt) supplemented with Keller and f/2 vitamins [37]. Control cultures were maintained in a 14:10 light:dark cycle at 85 μmol photon m^{-2} s^{-1} at a constant temperature of 18 °C (Table 1). Each population was grown in 20 mL media and each week, 200 μL was transferred to fresh media to ensure populations were always growing exponentially. Cultures were resuspended by gentle shaking every 2–3 days to prevent cells sticking to the bottom of the flask. For the evolution experiment, *O. tauri* populations were grown either in the control environment as described above, in low light, low phosphate, low salinity or high temperature (Table 1), or a changing environment (random) in which one of the environments from those listed was chosen at random at each transfer. We refer to the environments where the populations evolved as "selection environments". Populations were grown in the absence of viruses for 32 weeks, corresponding to approximately 200 generations.

Table 1. A comparison of the control environment and the treatments used for each selection environment used in this study.

Selection Environment	Control	Treatment
Light (μmol m^{-2} s^{-1})	85	60
Phosphate (μM)	10	5
Salinity (ppt)	30	25
Temperature (°C)	18	20

For the low light environment, culture flasks were wrapped in 0.15 neutral density foil to reduce light intensity. For the low phosphate environment, phosphate was reduced by preparing Keller medium with half the amount of β-glycerophosphate present in the control media. For low salt, Instant Ocean was added to reach a salinity of 25 ppt. Cultures in the high temperature environment were maintained on a heat mat (Exo Terra Heat Wave substrate heat mat, Yorkshire, UK) set at 20 °C. These selection environments were chosen so that the populations responded to them by changing their growth rates relative to the control environment—in batch culture rapid growth is favored by natural selection, so any environment that decreases growth rates should then result in natural selection for traits that will allow cell division rates to recover in that environment. However, the selection environments were not extreme, so that populations were still able to grow at a measurable rate and survive the dilution rate of the experiment. This is in part so that a similar number of generations elapse in all environments over the course of the experiment.

2.3. Testing RP Lines for Viral Production

All resistant producer (RP) lines were tested for viral production prior to the start of the experiment. To check whether the three producing lines (NG'10, NG'16 and NG27) were releasing infectious viruses, we used the supernatant to infect susceptible *O. tauri* strain RCC4221. Two milliliters

of each population were transferred to an Eppendorf tube and centrifuged at $8000 \times g$ for 15 min. Four hundred milliliters of the supernatant were removed carefully without drawing up any of the cells from the pellet at the bottom of the tube, and used to inoculate 1 mL of susceptible *O. tauri*. OtV5 was used as a positive control and Keller media was used as a negative control. Eight replicates were performed before the experiment was started. The test was performed every four weeks with three replicates per population. Samples were checked for lysis either by observing by eye whether they were green or clear, or by measuring cell densities using a BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer.

In addition to liquid lysis tests, frozen stocks of RP supernatant were made by adding dimethyl sulfoxide (DMSO) (final concentration 10%) and storing at -80 °C. We tested these samples for viruses using the plaque assay technique [34]. A 1.5% agarose suspension was made and 5 mL aliquots were prepared in Falcon tubes and held at 70 °C in a water bath. In a 50 mL Falcon tube, 30 mL exponentially growing *O. tauri* culture, 15 mL Keller media and 5 mL agarose were mixed rapidly but gently by inverting the tube (final agarose concentration 0.15%). The agarose was poured into a 12 cm square petri dish and left to set. Tenfold serial dilutions of the RP supernatant were made in 96-well plates using one row per sample. A Boekel Replicator was used to transfer all of the serial dilutions from one 96-well plate to one square petri dish. The replicator was sterilized between each use using ethanol and a flame. Petri dishes were checked daily for lysis plaques for a maximum of 10 days.

2.4. Testing Resistance Type Using OtV5 Inoculation

OtV5 inoculum was prepared prior to the start of the experiment and stored at -80 °C in 10% DMSO (final concentration) and inoculations were performed from the frozen stocks. The experiment did not include a co-evolving virus which allowed us to measure host evolution relative to the ancestral virus. After 32 weeks of evolution, each population was inoculated with a suspension of OtV5 particles to test whether it was susceptible or resistant to viral lysis. Samples were tested by inoculating 1 mL cell culture at a density of 10^5 with 10 μL OtV5 in 48-well plates with three replicates for each sample. Negative controls that were not inoculated with OtV5 were used as a comparison of cell growth. Cell density was measured using a FACSCanto flow cytometer 3 days after inoculation. Samples were run on 96-well plates by counting the total number of cells in 10 μL with a flow rate of 2.0 μL per second.

Data were analyzed with linear mixed effects models using the statistical packages lme4 [38] and lmerTest [39] in R (version 3.2.0, R Core Team, Vienna, Austria) to identify differences in cell densities after OtV5 inoculation compared to controls that were not inoculated. Selection environment, resistance type and treatment (inoculated or not inoculated) were set as fixed effects with population as a random effect. Post hoc Tukey tests were performed using lsmeans to confirm where significant differences occurred within the different effects.

2.5. Population Growth Rates, Cell Size and Cell Chlorophyll Content after Evolution

At the end of the evolution experiment, we quantified evolutionary responses by measuring average cell division rates and by measuring cell size and chlorophyll content for each population. All evolved populations were assayed in their selection environment and in the control environment, and all control populations were assayed in all selection environments except high temperature, since all populations in the high temperature environment went extinct and therefore there were no high temperature evolved strains. The populations that had evolved in a random environment for each transfer were only assayed in the control environment, which was not one of the environments they had been exposed to during the experiment, meaning only a correlated response (rather than a direct response) to selection could be obtained. Each population was assayed in triplicate. Due to the size of the experiment, assays were divided randomly into seven time blocks. This was factored into the statistical analysis.

Average cell division rates, which we refer to as "growth rates" are the average number of cell divisions per day over seven days, which corresponds to one transfer cycle. All populations were

first maintained in their assay environment for an acclimation period of one week, which was one full transfer cycle, prior to measuring growth rates. After acclimation, cells were counted using a FACSCanto flow cytometer before the transfer into the assay environment (to calculate the number of cells transferred into fresh media) and again after seven days of growth. Each sample was counted in triplicate. The cell counts were converted to cells per milliliter and the number of divisions per day was calculated using Equation (1).

$$\mu \left(d^{-1} \right) = \frac{\log_2 \left(\frac{N_t}{N_0} \right)}{t - t_0} \tag{1}$$

where μ is population growth rate, and N_t and N_0 are the cell densities (cells mL^{-1}) at times t and t_0 (days), respectively. This measures the average number of cell divisions per ancestor over a single growth cycle and allows a comparison of offspring production between environments even if there are differences in the shape of the population growth curve, or in cases where r cannot be accurately estimated. To avoid biases of cell divisions being dependent on the time of the cell cycle, cells were always measured at the same time of day (at the beginning of the light period when cells are in G1 phase).

Cell size was inferred from FSC (forward scatter), which was calibrated using beads of known sizes (1 μm, 3 μm and 6.6 μm). Chlorophyll fluorescence was inferred by measuring PerCP-Cy5.5 emission with excitation at 488 nm. Relative chlorophyll was analyzed by taking the average chlorophyll fluorescence for all susceptible strains in the control environment and setting this to a value of 1, with chlorophyll measurements of all other strains relative to this value.

Data were analyzed with linear mixed effects models. To analyze differences in growth rate, cell size and chlorophyll under different environments, selection environment, assay environment and resistance type were fixed effects and population and block ware random effects that were treated as un-nested. An additional model was fitted to examine whether there was a difference in growth rate when populations were assayed in their selection environment or when they were assayed in a different environment, with assay as the only fixed effect and population and block set as random effects.

2.6. Competition Assay

To measure competitive fitness, all evolved populations were competed against a green fluorescent protein (GFP) line of *O. tauri*. A Gateway enabled entry clone containing roGFP2 was obtained by linearizing pH2GW7-roGFP2 [40] with EcoRV. The linearized vector was recombined with pDONR207, creating a pDONR207-roGFP2 clone. A pOtOX binary vector [41] was adapted to become a Gateway® destination vector and pDON207-roGFP2 was recombined into the vector, downstream of the high-affinity phosphate transporter (HAPT) promoter [41]. The pOtOx-roGFP2 vector was subsequently transformed into *O. tauri* using the procedure previously described [42].
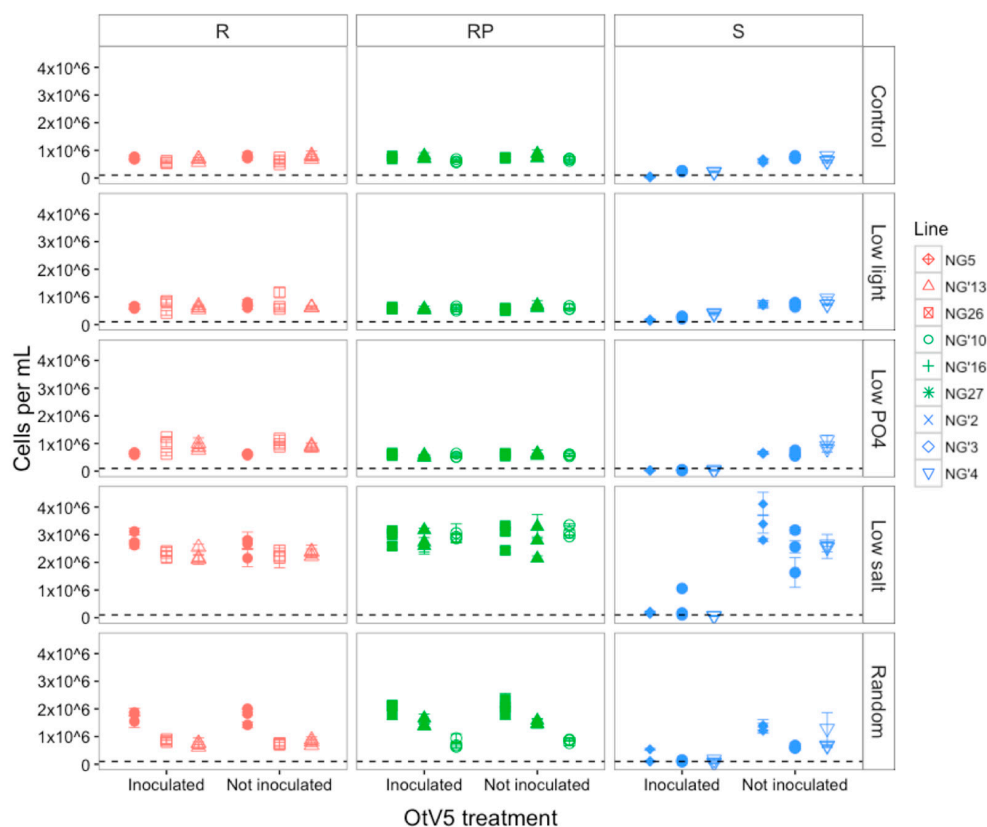
All evolved populations competed in the selection environment that they evolved in, and all control populations competed in the control environment as well as in each selection environment to measure plastic response. All of the random populations competed in the control environment. All populations, including the roGFP line, were acclimated for one week in the corresponding assay environment prior to the assay. Equal starting densities of 5×10^5 of each evolved population and the roGFP line were grown in 20 mL media for one week, after which cells were counted using a FACSCanto flow cytometer. GFP and non-GFP populations were distinguished by measuring fluorescein isothiocyanate A (FITC-A) emission at 519 nm with excitation at 495 nm. Competitiveness of the evolved populations was measured relative to the roGFP line as fold change in cell density. Data were analyzed with a linear mixed effects model, with selection environment, assay environment and resistance type as fixed effects and population and assay replicate as random effects.

3. Results

3.1. Susceptibility to OtV5 after Evolution

3.1.1. Host Resistance Type Was Maintained during Evolution

After 200 generations of evolution in the selection environments, all surviving R and RP populations remained resistant to OtV5 lysis and all S populations remained susceptible to viral lysis in those environments (Figure 1). A significant interaction between selection environment, resistance type and treatment (OtV5 inoculation) affected susceptibility of *O. tauri* to OtV5 (ANOVA environment × resistance type × treatment, $F_{8,238} = 15.22$, $p < 0.0001$). A post hoc Tukey test showed that this was due to cell lysis of S populations ($t_{8,238} = 10.66$, $p < 0.001$), whereas cell density of R and RP lines did not decrease compared to controls that were not inoculated. The highest cell densities were observed in the low salt (post hoc Tukey test, $t_{8,238} = -29.90$, $p < 0.0001$) and random (post hoc Tukey test, $t_{8,238} = -7.54$, $p < 0.0001$) environments. The OtV5-inoculated S populations in low phosphate were the only populations where cell density fell below the starting cell density across all populations, indicating almost complete cell lysis and no cell growth for this combination of resistance type and selection environment. R and RP lines did not show decreases in cell density after inoculation with OtV5 compared to controls that were not inoculated, whereas S lines did.


Figure 1. Mean (± SE) cell density mL^{-1} of resistant (R), resistant producer (RP) and susceptible (S) *O. tauri* lines three days after OtV5 inoculation in five environments. Points represent the average of the three assay replicates for each evolved population. Inoculated = populations inoculated with OtV5, Not inoculated = negative control populations that were grown for the same period without OtV5 inoculation. There were three evolved populations of each line. The dashed line represents the starting cell density at 100,000 cell mL^{-1}.

R and RP populations did not show a significant difference in cell density between populations that had been inoculated with OtV5 and populations that had not (Figure S1). In contrast, all S

populations inoculated with OtV5 showed a change in cell density relative to non-inoculated S populations in the same environments (ANOVA effect of resistance type on difference $F_{2,125} = 66.51$, $p < 0.0001$). The largest differences in cell densities between inoculated and non-inoculated populations were observed in S populations evolved in the low salt environment, showing that whilst all populations in this environment were able to reach high densities in the absence of viruses, they were unable to grow in the presence of OtV5 (Figure 1). The large difference in S populations in low salt was due to the high growth rate of populations that had not been inoculated, since inoculated populations did not fall to lower densities than inoculated S populations in any other environments.

3.1.2. OtV5-Mediated Lysis Decreased in Susceptible Populations

Although S populations remained sensitive to viral lysis at the end of the evolution experiment, complete lysis was not observed in all populations, with a small proportion of populations able to reach numbers above the starting density of 100,000 cells mL^{-1} (Figure 1). This was in contrast to the beginning of the evolution experiment, when all susceptible populations fell below 100,000 cells mL^{-1} after inoculation with OtV5, indicating near-complete lysis (ANOVA effect of time point on cell density, $F_{1,65} = 21.87$, $p < 0.0001$) (Figure 2). The highest proportion of S cells that did not lyse was found in low salt evolved populations, suggesting that resistance mutations had been maintained in this environment, despite no selection by OtV5. To eliminate the possibility that the infection dynamics had changed and that the population decline was still in process, we measured the population density seven days after inoculation and did not observe any further decrease in population density (Figure S2).
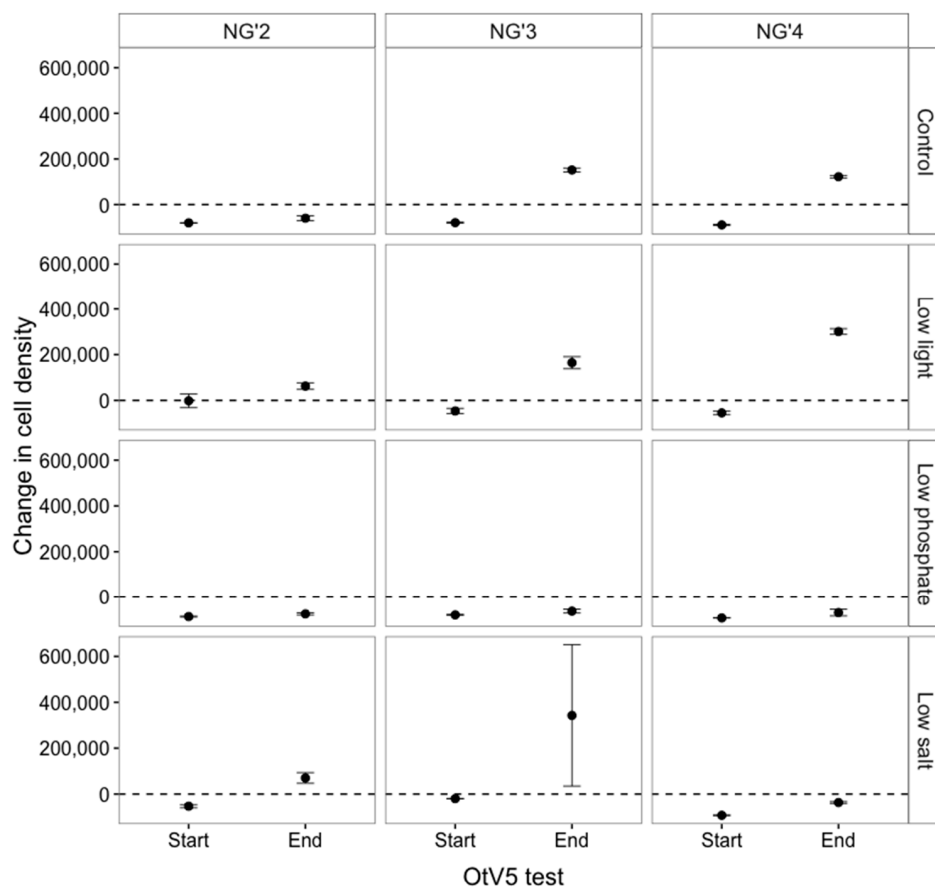
Figure 2. Change in cell density of the susceptible lines NG′2, NG′3 and NG′4 after OtV5 inoculation one week into the selection experiment (Start) and after 32 transfer cycles of evolution (End). The dashed line represents no change.

3.1.3. RPs Stopped Producing Viruses Early in the Evolution Experiment

During the evolution experiment, RP populations (NG27, NG'10 and NG'16) were tested to check that they were still producing viruses. Seven transfer cycles into the evolution experiment, all NG27 populations in all environments were still producing infectious viruses, as observed by cell lysis when their supernatant was used to inoculate the susceptible *O. tauri* strain RCC4221. In contrast, RCC4221 cultures that were inoculated with the supernatant of all populations of NG'10 and NG'16 continued growing, showing that no observable lysis had occurred. After 17 transfers in the selection environments, all RP populations in all environments had stopped producing infectious viruses (Figure S3), as observed by flow cytometric cell counts of RCC4221 populations inoculated with the supernatant of RP populations. When it was clear that all RP populations had stopped producing infectious viruses, frozen supernatant samples collected at transfers 9, 12, 14 and 15 were tested using the plaque assay method. No plaques were observed in any samples tested, thus we concluded that all RP populations in all environments had stopped producing viruses within nine weeks of the selection experiment.

3.2. Changes in Trait Values after Evolution

3.2.1. Changes in Cell Division Rate and Population Persistence during the Selection Experiment

Here, we focus on how growth rates vary with resistance type, selection environment and the number of transfer cycles in the selection environment. Growth rates of all populations were measured as the number of cell divisions per day, at four time points during the experiment (including at the beginning and end) (Figure 3). When comparing these time points, growth was significantly affected by environment, resistance type and time point ($p < 0.0001$ for all effects). In the first transfer cycle, which measured the population growth rates at the very start of the experiment following one week of acclimation, two out of the three RP lines (NG'10 and NG'16) had increased growth rates across all environments except for low phosphate (ANOVA effect of growth rate on cell divisions, $F_{3,5} = 17.19$, $p = 0.046$). These results are reported in [43].

After 14 transfer cycles, growth rates of all populations were approximately one division per day in the high salt, low phosphate, low light and random environments (Figure 3). In the control environment, growth rate varied across all S lines, even between populations of the same starting line, ranging from 0.18 to 0.87 divisions per day. The increased growth of all lines evolving in low phosphate to one division per day, which is the normal growth rate reported for *O. tauri* in phosphate-replete media, is consistent with adaptation to low phosphate in less than 100 generations. Additionally, RP lines that had been dividing more rapidly at transfer 1 were dividing at the same rate as other lines within each environment (Figure 3). This may be because the RP populations had stopped producing viruses and shifted to the R resistance type (see Section 3.1.3), thereby losing the growth advantage associated with the RP resistance type early on in this experiment. By transfer 24, all populations in the high temperature environment had gone extinct. RP populations went extinct more quickly than S and R populations, with 66% of RP lines extinct by T14 compared to 33% and 22% of S and R, respectively (Figure 3). At transfer 20, only three high temperature populations remained: one S (NG'4) and two R (NG'13 and NG26).
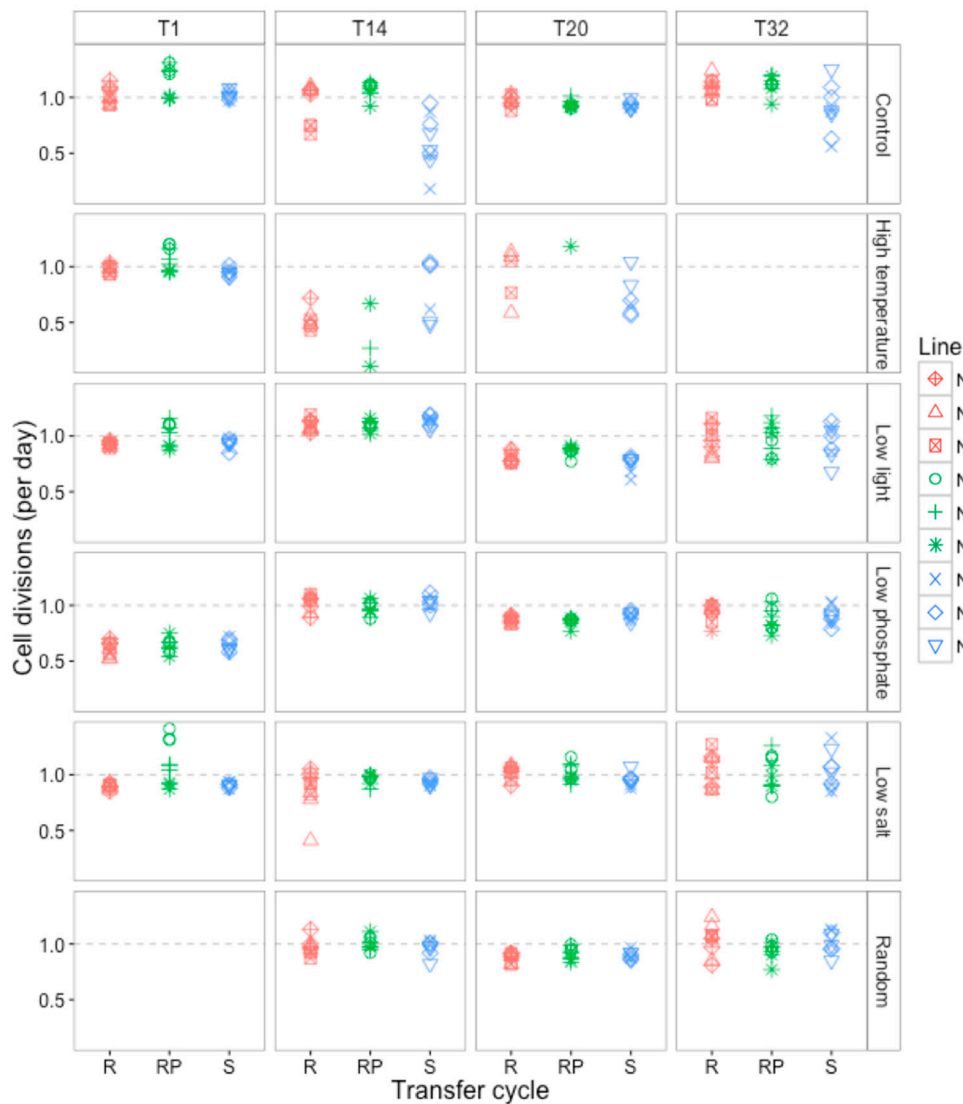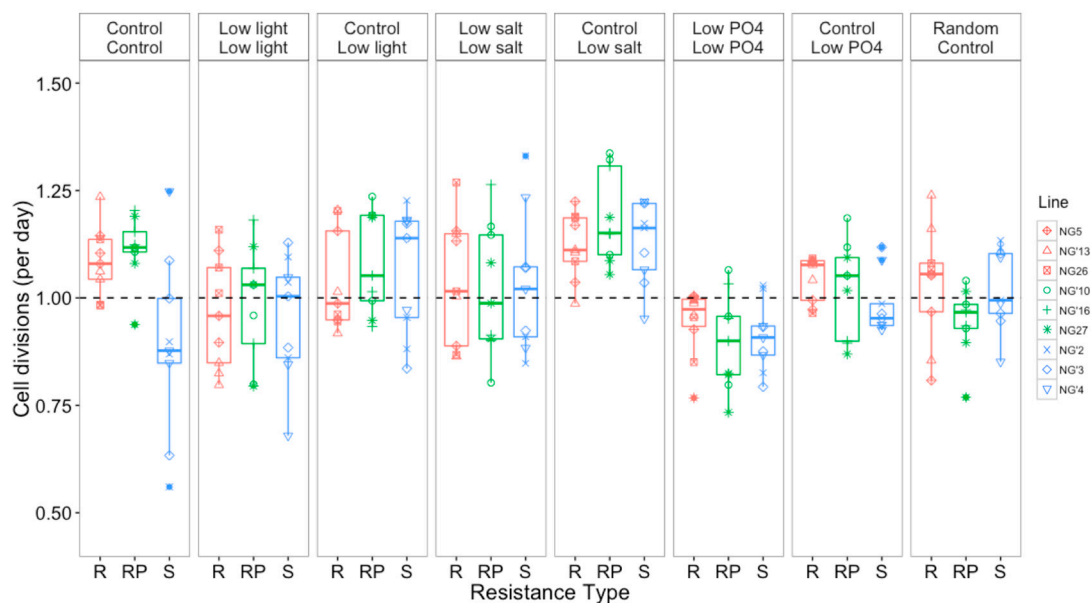
Figure 3. Growth rates as measured by mean cell divisions per day for each evolving population over four time points (1, 14, 20 and 32 transfer cycles). The dashed line represents one cell division per day. T1 is the growth rate following acclimation at the beginning of the experiment. There are no growth measurements for the randomized environment at T1 because lines had only been growing for one transfer cycle.

3.2.2. Growth Rates Varied with Selection Environment and Assay Environment after Evolution

After approximately 200 generations of evolution in each environment, a transplant assay was performed to quantify environmental effects on population growth rate, cell size and cell chlorophyll content for each evolved population. Here we define the selection environment as the environment that the population evolved in, and the assay environment as the environment in which measurements were taken. The direct response to selection compares the growth rate of a population evolved in a given selection environment with the growth rate of a population evolved in the control environment when both are grown (separately) in that given selection environment. The effect of selection environment on the direct response to evolution was large, and driven by the direct response to selection in the low phosphate environment (ANOVA effect selection environment on direct response, $F_{2,228} = 9.26$, $p = 0.0001$), whereas the effect of resistance type was smaller (ANOVA effect of resistance type on direct response, $F_{2,228}$ 2.87, $p = 0.06$).

Selection environment alone and assay environment alone both had a significant effect on population growth rate (ANOVA effect of selection environment on growth, $F_{4,200} = 19.92$, $p < 0.0001$; ANOVA effect of assay environment on growth, $F_{3,758} = 32.43$, $p < 0.0001$), which shows that environment affected growth rates. Resistance type also had an effect on growth rate (ANOVA effect of resistance type on growth, $F_{2,195} = 4.21$, $p = 0.02$), with R populations having the fastest cell division rates and S populations having the slowest cell division rates. Additionally, an interaction between selection environment and assay environment affected growth rate, indicating that the way in which selection environment affected growth differed between assay environments (ANOVA selection environment × assay environment, $F_{3,757} = 2.89$, $p = 0.03$). The fastest growth rates were seen in the evolved control populations that were assayed in low salt (Figure 4). Better performance was not due to being assayed in the same selection environment that the populations had evolved in (ANOVA effect of being assayed in selection environment on growth, $F_{1,831} = 1.70$, $p = 0.19$).

Figure 4. Mean cell divisions per day (±SEM). R = resistant, RP = resistant producer, S = susceptible. Each panel represents a growth assay, with cells evolved in the selection environment (top label) and growth rates measured in the assay environment (bottom label). The dashed line indicates, for reference, one cell division per day.

### 3.2.3. Resistance Type Affected Cell Size and Chlorophyll Content

Cells from different resistance types had different cell sizes (ANOVA effect of resistance type on size, $F_{2,140} = 9.49$, $p = 0.0001$) (Figure S4) and this was not affected during evolution in any of the environments (ANOVA effect of selection environment on size, $F_{4,155} = 0.66$, $p = 0.62$; ANOVA effect of assay environment on size, $F_{3,735} = 1.60$, $p = 0.19$). The greatest variation in cell size between populations was observed when control-evolved cells were assayed in low salt (0.86–0.97 μm) across all resistance types. Less variation was found in the control-evolved cells assayed in low phosphate (0.82–0.97 μm).

The environment in which populations were assayed had a significant effect on the relative chlorophyll content per cell volume (ANOVA effect of assay environment on chlorophyll, $F_{3,744} = 17.83$, $p < 0.0001$). However, selection environment did not (ANOVA effect of selection environment on chlorophyll, $F_{4,168} = 0.90$, $p = 0.47$). Resistance type affected chlorophyll content (ANOVA effect of resistance type on chlorophyll, $F_{2,153} = 8.54$, $p < 0.0001$). Susceptible populations that had been evolving in the control environment contained high amounts of chlorophyll relative to their cell size when assayed under all three selection environments (low light, low salt and low phosphate) (Figure S5).

3.3. Selection and Assay Environments Affect Competitive Ability of O. tauri

In addition to measuring growth rate, size and chlorophyll content, we also tested if costs of resistance could be observed during pairwise competition between each population of S, R, and RP. We measured relative competitive ability, by competing each population against a common competitor harboring a GFP reporter, which allowed us to distinguish between the evolved population and the GFP line. Both selection environment and assay environment affected competitive ability against a roGFP-labeled strain (ANOVA effect of selection environment on competitiveness, $F_{4,622} = 16.41$, $p = < 0.0001$; ANOVA effect of assay environment on competitiveness, $F_{3,622} = 10.96$, $p < 0.0001$). Most populations were poor competitors relative to the roGFP line (Figure 5). Lines evolved in low light and low salt were the best competitors. Lines that were assayed in the same environment that they had evolved in were better competitors than control lines that were assayed in the selection environments. This shows that these lines adapted to their selection environment and that growth rate is not necessarily the most appropriate measure of adaptation in this study, which is consistent with other studies in *Ostreococcus* spp. [44]. Interestingly, populations in the control environment were the worst competitors, regardless of resistance type, with a 0.56 mean fold change, showing that all populations were out-competed by the roGFP line. This indicates that the control environment did in fact exert less selection on the populations than did the other environments.

Resistance type alone did not affect competitive ability (ANOVA effect of resistance type of competitiveness, $F_{2,622} = 1.22$, $p = 0.30$). Although competitive ability differed between resistance types, the response was not consistent across assay environments, with no one resistance type consistently being a better or poorer competitor.
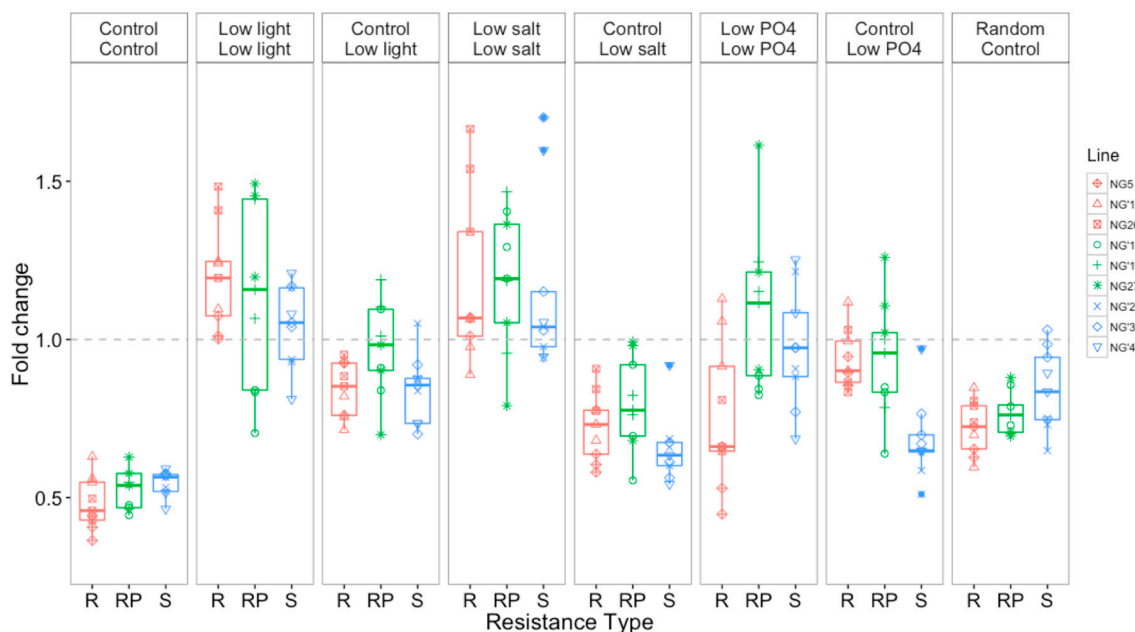
Figure 5. Competitive ability, as measured by fold difference in growth relative to a roGFP-modified *O. tauri* line, of evolved populations and control populations assayed in the selection environments. R = resistant, RP= resistant producer, S = susceptible. Each panel represents one assay, with populations evolved in the selection environment (top label) and competitiveness measured in the assay environment (bottom label). The dashed line represents no change (i.e., equal proportions of roGFP and competitor populations).

4. Discussion

We examined whether cost of resistance varied with the abiotic environment in which *O. tauri* populations evolved. A cost of resistance can manifest in different ways depending on the interaction

between host and virus and on the way in which resistance is acquired (e.g., entry of the virus into the cell, and ability of the virus to replicate within the cell and cause lysis). This means that it is often difficult to detect a cost of resistance, so we measured three host responses: ability to maintain resistance, population growth rate and competitive ability.

4.1. Susceptibility to OtV5 Did Not Change after Evolution

After evolution in a new environment, OtV5 was still able to lyse susceptible (S) *O. tauri* populations under all environmental conditions tested, whereas R and RP populations remained resistant under all environments, despite the absence of selection pressure for viral resistance (Figure 1). Resistance to pathogens often comes at a fitness cost, such that a proportion of susceptible individuals remain in the population, thereby allowing viruses to persist [21]. If resistance does carry a fitness cost, populations should revert to susceptibility over time, in the prolonged absence of viruses, even if that cost is low, because susceptible cells have a fitness advantage in the absence of viruses [29]. Our study indicated that if there is a cost to simply maintaining resistance in *O. tauri*, it is small. Over the time scale of our experiment, the fitness advantage of susceptible types in the absence of viruses would have to be about 0.005 for a mutation conferring susceptibility in a resistant background to be fixed in the population following a spontaneous reversion of a resistant cell (where we calculate s from $\frac{1}{2}s/(1-e^{-2sN})$, and assume a starting frequency of 1/N [45]).

It is possible that there is a genetic constraint preventing the loss of resistance, making the transition from resistant to susceptible phenotypes rare even if resistance is costly. This is consistent with recent studies showing that the resistance mechanism in *O. tauri* is an intracellular response [35] and probably also involves rearrangements of chromosome 19 [36]. The presence of a genetic constraint on losing resistance would favor compensatory mutations that lead to alleles being selected that reduce the cost of resistance [46,47]. Studies evolving *E. coli* in the absence of bacteriophage observed that the cost of resistance to the T4 bacteriophage decreased after 400 generations due to compensatory adaptations [46]. A second possibility is that the cost of resistance to one strain of OtV means increased susceptibility to other virus strains. For example, cyanobacteria can rapidly evolve viral resistance when coevolving with viruses, however increased resistance to one virus can lead to a narrower resistance range thereby making cells more susceptible to other virus strains [27,28]. *O. tauri*-virus interactions can be complex with some OtVs being very specific to host *O. tauri* strains while others are generalists that can infect many strains [26,48]. Our experiment focused only on OtV5 and did not examine evolution of host resistance range.

At the end of the evolution experiment, OtV5 lysed susceptible (S) populations in all environments, but the extent of lysis differed between environments (Figure 1). This could be because one or more resistance mutations had appeared and risen to a detectable frequency in some populations. It is unclear whether incomplete lysis was due to some resistant cells evolving in the susceptible populations, or whether susceptible populations had evolved to make virus entry harder but still possible. Inoculations were performed from frozen stocks, thus OtV5 was not coevolving with the host, enabling us to measure evolution in the *O. tauri* populations relative to the ancestral virus population. We cannot rule out the possibility that there was a slow loss of infective virus titer in the cryopreserved stock, leading to fewer infectious viruses in the inoculum and therefore a lower multiplicity of infection. Physiological changes in susceptible populations arising as an adaptive response to abiotic environmental change did not prevent viral lysis, indicating that viral adsorption was not completely inhibited. This was even evident in the control populations, suggesting that although these populations did not experience a change in environment, they may have evolved changes in cell surface proteins, since were still evolving for the full length of the experiment. However, the biotic environment plays a larger role in resistance acquisition, since resistance to viruses is selected for by the virus [49]. Chemostat experiments to monitor population dynamics in *Chlorella* and *Paramecium bursaria Chlorella* Virus 1 (PBCV-1) showed that control populations maintained in the absence of viruses did not evolve resistance to the ancestor virus, suggesting that resistance arises from host-virus interactions [23]. In contrast, sensitive *E. coli*

cells evolved complete resistance to λ infection and resistant cells increased susceptibility to T6* infection after 45,000 generations in the absence of phage [29]. In our experiment, low phosphate was the only environment in which the cell numbers of all lines fell below the starting cell density (Figure 1), suggesting that this environment either affected the infectivity of OtV5 directly or the cells' response to infection. Other studies report the opposite, with reduced virus infection of algae under low phosphate, possibly due to the requirement of phosphate for viral replication [9,10,13]. Though phosphate levels were low in our experiment, they were sufficient for population growth to be positive, and were higher than found in the Mediterranean Sea [50]. Conflicting results highlight the complexity of host-virus interactions in different study systems as well as different growth conditions.

There was a selection pressure against viral production on RP lines, but not on host resistance across all RP lines in all environments. Similarly, Yau et al. reported that over a two year period RP populations maintained under standard laboratory conditions stopped producing viruses [36]. If RP populations are indeed made up of a majority of resistant cells with a small proportion of susceptible cells arising that lyse upon OtV5 infection, thus maintaining the production of viruses in the media, then we would expect resistance to be selected for in the presence of viruses. Resistance in *O. tauri* is expected to be caused by over-expression of glycosyltransferase genes on chromosome 19 [36]. In this study, the selection environment did not affect the time it took for a selective sweep of resistance to occur in the RP lines, supporting the conclusion that there was little or no selection against resistance, that there is a genetic constraint on losing resistance, or that compensatory mutations enabled resistance to be maintained.

4.2. Resistance Type and Environment Affect Evolutionary Response of O. tauri to Environmental Change

We did not observe a growth cost of *O. tauri* being resistant to viral lysis, since R populations had the fastest growth overall whereas S populations had the slowest growth. Data on the growth effects of resistance in marine algae are rare. A 20% reduction in growth was reported in the ubiquitous cyanobacterium *Synechococcus* [22], however it is unknown whether viral resistance generally carries a growth cost in eukaryotic algae. Even with no or minimal costs of resistance, the chromosomal rearrangement associated with resistance in *O. tauri* means that the different resistance types have different genetic backgrounds. Therefore, evolution could take different trajectories in hosts with different resistance types due to epistatic interactions between resistance and adaptive changes. For example, trade-off shape varied in response to environmental change and physiological changes of bacteriophage resistant *E. coli*, leading to variation in sensitivity to environmental change across different strains [51]. In our study, when considering the direct response to evolution (which compares the growth rate of the evolved population in its selection environment with the plastic response of the control line in that selection environment), resistance type did not drive direct response. This indicates that the growth response of the three resistance types was similar within environments. If there is an effect of genetic background being introduced by resistance, it is not evident at the level of growth rate under these conditions.

Selection environment affected population growth, with populations evolved in the control environment having the highest growth rates in all assay environments (Figure 4). The decrease in growth in response to our selection environments is consistent with them being of lower quality than the control environment, by design, so that selection was stronger in the non-control environments. Variation in the direct response to evolution was explained by selection environment. Populations evolved in low phosphate had the lowest growth, which is expected when cells are nutrient limited. Interestingly, populations that had evolved in the control environment grew more rapidly in low phosphate than populations that had evolved in low phosphate. This may be because populations that had been evolving in the control environment had enough phosphate reserves within the cell to grow normally for a short period, since growth was only assayed for seven days. Overall, growth rates of populations evolved in the control environment were greater when assayed in the selection environments than the populations that had evolved in those environments, showing

that increased growth could be initiated as a stress response, and that cells in the control environment (which was nutrient-replete, and at the optimal temperature and usual salinity for these lines of *O. tauri*) were in better condition overall. The extent of a cost of resistance can be highly dependent on environment. For example, cost of resistance differs when fitness of *E. coli* is measured under different nutrient resources and concentrations [20,52]. We show here that growth rate measurements may not be sensitive enough to detect very small differences between populations conferring a cost of resistance in *O. tauri*, as has also been observed in short term experiments using a single [35] and multiple environments [43]. Studies in bacteria also found that resistant strains grew at the same rate as susceptible strains [21,46]. Our results indicate that, regardless of resistance type, *O. tauri* is able to adapt to environmental change including low light, low salt and low phosphate. However, all populations in the high temperature environment went extinct, despite the modest (2 °C) increase, suggesting that although *O. tauri* can tolerate and grow at higher temperatures over the short-term, sustained temperature increases may exert stronger selection than predicted from short-term studies. It is not possible to infer as of yet whether resistance affects growth rate in natural habitats or whether a cost of resistance is instead associated with tradeoffs that are not related to the abiotic environment, such as resistance to other viral strains.

In contrast to cell division rates, resistance type affected cell size and chlorophyll content, but selection environment did not. Cells in RP populations were sometimes larger in size and S populations were slightly smaller. Often, small size is associated with a response to nutrient limitation, increased temperature and light limitation in phytoplankton [53–57], however all lines in this study showed slightly increased cell size in low phosphate. An increased cell volume has been observed in coccolithophores in response to phosphate limitation suggesting the adaptive strategy is to reduce phosphorous requirements rather than increasing surface area to volume ratio [58].

RP populations had less chlorophyll in most environments, however overall there was substantial variation in chlorophyll content, especially in S populations. When assayed in the control environment, populations that had evolved in low light, low salt, low phosphate and the random environment had lower chlorophyll than did control populations assayed in these same environments. The response of populations evolved in the control environment increasing their relative chlorophyll content when assayed in low light is consistent with responses to light limitation in other green algae [59–61]. Here, we show that response of chlorophyll content to environmental change is variable, both with environment and with resistance type. Previous studies in marine microalgae have reported lower reduced chlorophyll content under nutrient limitation [62] and higher chlorophyll content under some optimal salinities [63,64].

4.3. Resistance Type Did Not Affect Competitive Ability Regardless of Environment

Reduced competitive ability is often one of the main restrictions for resistance spreading through a population, however resistance type did not affect the competitive ability of evolved populations in our experiment. We found that environment did affect competitive ability, and similarly in bacteria, the environment that populations evolve in, such as the limiting sugar source or spatial heterogeneity, can affect competitive ability, both with and without coevolving phage [51,52,65]. Other studies have reported a trade-off between competitive ability and resistance, whereas here we found no evidence for reduced resistance with increased competitive ability. The nature of a cost of resistance will depend on the genetic or physiological changes to the cell. For example, *E. coli* mutants showed high variability in competitiveness which was associated with resistance strategy, with cross-resistance to phage T7 significantly decreasing competitive fitness by approximately 3-fold [21]. In contrast, competitions with cyanobacteria showed that total resistance (the total number of viruses to which a host strain was resistant) did not affect competitive ability [22]. These examples reveal that the magnitude of the reduced competitiveness trade-off can depend on the specific resistance strategy.

Evolved populations in the non-control environments were better competitors than control populations that had been exposed to the selection environments for the first time (plastic response),

indicating that all lines had adapted to their selection environment. Thus, growth rate is not the most appropriate measure of adaptation in *O. tauri*, since the plastic response was to increase population growth rates, and the evolutionary response was to reverse this plastic increase in growth rates, and this strategy was associated with an increase in competitive fitness. Similar results have been reported previously in *Ostreococcus* spp. where populations with high growth rates in monoculture were poorer competitors than those with lower growth rates in monoculture [44].

5. Conclusions

Here, we show that there was no detectable cost of resistance to OtV5 as measured by growth rate or competitive ability for *O. tauri* evolved in several different environments, and that resistance to viruses did not affect adaptation to environmental change. Additionally, we found no reversion of R or RP populations to S as tested by exposure to OtV5, whereas lysis occurred in all S populations. Additionally, all RP lines stopped producing viruses within nine weeks of the experiment. This suggests that a shift from susceptibility to resistance is more common than a shift from resistance to susceptibility, regardless of selection environment, at least for the range of environments used here. Our experiment shows that the conditions under which a cost of resistance may occur or affect adaptation in *O. tauri* are not clear in the laboratory. More work is needed to understand the factors that affect host–virus interactions in the marine environment to better understand evolutionary and ecological responses of marine eukaryotic microalgae to environment change.

Author Contributions: S.E.H., P.F.V. and S.C. conceived and designed the experiments; S.E.H. and K.K. performed the experiments; and S.E.H., P.F.V. and S.C. analyzed the data. All authors contributed to writing the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Suttle, C.A. Marine viruses—Major players in the global ecosystem. *Nat. Rev. Microbiol.* **2007**, *5*, 801–812. [CrossRef] [PubMed]
2. Wilhelm, S.W.; Suttle, C.A. Viruses and nutrient cycles in the sea. *Bioscience* **1999**, *49*, 781–788. [CrossRef]
3. Koskella, B.; Brockhurst, M.A. Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* **2014**, *38*, 916–931. [CrossRef] [PubMed]
4. Dennehy, J.J. What Can Phages Tell Us about Host-Pathogen Coevolution? *Int. J. Evol. Biol.* **2012**, *2012*, 396165. [CrossRef] [PubMed]
5. Flint, S.J.; Enquist, L.W.; Krug, L.; Racaniella, V.; Skalka, A. *Principles of Virology: Molecular Biology, Pathogenesis, Virus Ecology*, 4th ed.; ASM Press: Washington, DC, USA, 2000.
6. Doney, S.C.; Ruckelshaus, M.; Emmett Duffy, J.; Barry, J.P.; Chan, F.; English, C.A.; Galindo, H.M.; Grebmeier, J.M.; Hollowed, A.B.; Knowlton, N.; et al. Climate Change Impacts on Marine Ecosystems. *Ann. Rev. Mar. Sci.* **2012**, *4*, 11–37. [CrossRef] [PubMed]

7. Nagasaki, K.; Yamaguchi, M. Effect of temperature on the algicidal activity and the stability of HaV (Heterosigma akashiwo virus). *Aquat. Microb. Ecol.* **1998**, *15*, 211–216. [CrossRef]

8. Wells, L.E.; Deming, J.W. Effects of temperature, salinity and clay particles on inactivation and decay of cold-active marine Bacteriophage 9A. *Aquat. Microb. Ecol.* **2006**, *45*, 31–39. [CrossRef]

9. Bellec, L.; Grimsley, N.; Derelle, E.; Moreau, H.; Desdevises, Y. Abundance, spatial distribution and genetic diversity of *Ostreococcus tauri* viruses in two different environments. *Environ. Microbiol. Rep.* **2010**, *2*, 313–321. [CrossRef] [PubMed]

10. Bratbak, G.; Egge, J.K.; Heldal, M. Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms. *Mar. Ecol. Prog. Ser.* **1993**, *93*, 39–48. [CrossRef]

11. Bratbak, G.; Jacobsen, A.; Heldal, M.; Nagasaki, K.; Thingstad, F. Virus production in *Phaeocystis pouchetii* and its relation to host cell growth and nutrition. *Aquat. Microb. Ecol.* **1998**, *16*, 1–9. [CrossRef]

12. Wilson, W.H.; Carr, N.G.; Mann, N.H. The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium *Synechococcus* sp. WH7803. *J. Phycol.* **1996**, *32*, 506–516. [CrossRef]

13. Maat, D.; Crawfurd, K.; Timmermans, K.; Brussard, C. Elevated CO_2 and phosphate limitation favor *Micromonas pusilla* through stimulated growth and reduced viral impact. *Appl. Environ. Microbiol.* **2014**, *80*, 3119–3127. [CrossRef] [PubMed]

14. Jacquet, S.; Bratbak, G. Effects of ultraviolet radiation on marine virus-phytoplankton interactions. *FEMS Microbiol. Ecol.* **2003**, *44*, 279–289. [CrossRef]

15. Jacquet, S.; Heldal, M.; Iglesias-Rodriguez, D.; Larsen, A.; Wilson, W.; Bratbak, G. Flow cytometric analysis of an *Emiliana huxleyi* bloom terminated by viral infection. *Aquat. Microb. Ecol.* **2002**, *27*, 111–124. [CrossRef]

16. Thyrhaug, R.; Larsen, A.; Brussaard, C.P.D.; Mcfadden, P. Cell Cycle Dependent Virus Production in Marine Phytoplankton 1. *Cell Cycle* **2002**, *343*, 338–343.

17. Larsen, J.B.; Larsen, A.; Thyrhaug, R.; Bratbak, G.; Sandaa, R.-A. Response of marine viral populations to a nutrient induced phytoplankton bloom at different pCO$_2$ levels. *Biogeosci. Discuss.* **2007**, *4*, 3961–3985. [CrossRef]

18. Chen, S.; Gao, K.; Beardall, J. Viral attack exacerbates the susceptibility of a bloom-forming alga to ocean acidification. *Glob. Chang. Biol.* **2014**, *21*, 629–636. [CrossRef] [PubMed]

19. Danovaro, R.; Corinaldesi, C.; Dell'Anno, A.; Fuhrman, J.A.; Middelburg, J.J.; Noble, R.T.; Suttle, C.A. Marine viruses and global climate change. *FEMS Microbiol. Rev.* **2011**, *35*, 993–1034. [CrossRef] [PubMed]

20. Bohannan, B.J.M.; Kerr, B.; Jessup, C.M.; Hughes, J.B.; Sandvik, G. Trade-offs and coexistence in microbial microcosms. *Antonie van Leeuwenhoek* **2002**, *81*, 107–115. [CrossRef] [PubMed]

21. Lenski, R.E. Experimental Studies of Pleiotropy and Epistasis in *Escherichia coli*. I. Variation in Competitive Fitness Among Mutants Resistant to Virus T4. *Evolution* **1988**, *42*, 425–432. [CrossRef]

22. Lennon, J.T.; Khatana, S.A.M.; Marston, M.F.; Martiny, J.B.H. Is there a cost of virus resistance in marine cyanobacteria? *ISME J.* **2007**, *1*, 300–312. [CrossRef] [PubMed]

23. Frickel, J.; Sieber, M.; Becks, L. Eco-evolutionary dynamics in a coevolving host-virus system. *Ecol. Lett.* **2016**, *19*, 450–459. [CrossRef] [PubMed]

24. Seed, K.D.; Faruque, S.M.; Mekalanos, J.J.; Calderwood, S.B.; Qadri, F.; Camilli, A. Phase Variable O Antigen Biosynthetic Genes Control Expression of the Major Protective Antigen and Bacteriophage Receptor in Vibrio cholerae O1. *PLoS Pathog.* **2012**, *8*, e1002917. [CrossRef] [PubMed]

25. León, M.; Bastías, R. Virulence reduction in bacteriophage resistant bacteria. *Front. Microbiol.* **2015**, *6*, 1–7. [CrossRef] [PubMed]

26. Clerissi, C.; Desdevises, Y.; Grimsley, N. Prasinoviruses of the marine green alga *Ostreococcus tauri* are mainly species specific. *J. Virol.* **2012**, *86*, 4611–4619. [CrossRef] [PubMed]

27. Marston, M.F.; Pierciey, F.J.; Shepard, A.; Gearin, G.; Qi, J.; Yandava, C.; Schuster, S.C.; Henn, M.R.; Martiny, J.B.H. Rapid diversification of coevolving marine Synechococcus and a virus. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4544–4549. [CrossRef] [PubMed]

28. Avrani, S.; Wurtzel, O.; Sharon, I.; Sorek, R.; Lindell, D. Genomic island variability facilitates *Prochlorococcus*-virus coexistence. *Nature* **2011**, *474*, 604–608. [CrossRef] [PubMed]

29. Meyer, J.R.; Agrawal, A.A.; Quick, R.T.; Dobias, D.T.; Schneider, D.; Lenski, R.E. Parallel changes in host resistance to viral infection during 45,000 generations of relaxed selection. *Evolution* **2010**, *64*, 3024–3034. [CrossRef] [PubMed]

30. Avrani, S.; Lindell, D. Convergent evolution toward an improved growth rate and a reduced resistance range in *Prochlorococcus* strains resistant to phage. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E2191–E2200. [CrossRef] [PubMed]

31. Avrani, S.; Schwartz, D.A.; Lindell, D. Virus-host swinging party in the oceans: Incorporating biological complexity into paradigms of antagonistic coexistence. *Mob. Genet. Elem.* **2012**, *2*, 88–95. [CrossRef] [PubMed]

32. Field, C.B. Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science* **1998**, *281*, 237–240. [CrossRef] [PubMed]

33. Bellec, L.; Grimsley, N.; Moreau, H.; Desdevises, Y. Phylogenetic analysis of new Prasinoviruses (*Phycodnaviridae*) that infect the green unicellular algae *Ostreococcus*, *Bathycoccus* and *Micromonas*. *Environ. Microbiol. Rep.* **2009**, *1*, 114–123. [CrossRef] [PubMed]

34. Derelle, E.; Ferraz, C.; Escande, M.-L.; Eychenié, S.; Cooke, R.; Piganeau, G.; Desdevises, Y.; Bellec, L.; Moreau, H.; Grimsley, N. Life-cycle and genome of OtV5, a large DNA virus of the pelagic marine unicellular green alga *Ostreococcus tauri*. *PLoS ONE* **2008**, *3*, e2250. [CrossRef] [PubMed]

35. Thomas, R.; Grimsley, N.; Escande, M.-L.; Subirana, L.; Derelle, E.; Moreau, H. Acquisition and maintenance of resistance to viruses in eukaryotic phytoplankton populations. *Environ. Microbiol.* **2011**, *13*, 1412–1420. [CrossRef] [PubMed]

36. Yau, S.; Hemon, C.; Derelle, E.; Moreau, H.; Piganeau, G.; Grimsley, N. A Viral Immunity Chromosome in the Marine Picoeukaryote, *Ostreococcus tauri*. *PLoS Pathog.* **2016**, *12*, e1005965. [CrossRef] [PubMed]

37. Keller, M.D.; Selvin, R.C.; Claus, W.; Guillard, R.R.L. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* **1987**, *23*, 633–638.

38. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting Linear Mixed-Effects Models using lme4. *J. Stat. Softw.* **2014**, *67*, 51.

39. Kuznetsova, A.; Brockhoff, P.B.; Christensen, R.H.B. lmerTest: Tests in Linear Mixed Effects Models. Cran, R Package. 2015. Available online: http://CRAN.R-project.org/package=lmerTest (accessed on 15 January 2017).

40. Schwarzländer, M.; Fricker, M.; Müller, C.; Marty, L.; Brach, T.; Novak, J.; Sweetlove, L.; Hell, R.; Meyer, A. Confocal imaging of glutathione redox potential in living plant cells. *J. Microsc.* **2008**, *231*, 299–316. [CrossRef] [PubMed]

41. Corellou, F.; Schwartz, C.; Motta, J.-P.; Djouani-Tahri, E.B.; Sanchez, F.; Bouget, F.-Y. Clocks in the green lineage: Comparative functional analysis of the circadian architecture of the picoeukaryote *Ostreococcus*. *Plant Cell* **2009**, *21*, 3436–3449. [CrossRef] [PubMed]

42. Van Ooijen, G.; Knox, K.; Kis, K.; Bouget, F.-Y.; Millar, A.J. Genomic Transformation of the Picoeukaryote *Ostreococcus tauri*. *J. Vis. Exp.* **2012**, *65*, e4074. [CrossRef] [PubMed]

43. Heath, S.E.; Collins, S. Mode of resistance to viral lysis affects host growth across multiple environments in the marine picoeukaryote *Ostreococcus tauri*. *Environ. Microbiol.* **2016**, *18*, 4628–4639. [CrossRef] [PubMed]

44. Schaum, E.; Collins, S. Plasticity predicts evolution in a marine alga. *Proc. R. Soc. B* **2014**, *281*. [CrossRef] [PubMed]

45. Bell, G. *Selection: The Mechanism of Evolution*, 2nd ed.; Oxford University Press: Oxford, UK, 2008.

46. Lenski, R.E. Experimental Studies of Pleiotropy and Epistasis in *Escherichia coli*. II. Compensation for Maldaptive Effects Associated with Resistance to Virus T4. *Evolution* **1988**, *42*, 433–440. [CrossRef]

47. Björkman, J.; Nagaev, I.; Berg, O.G.; Hughes, D.; Andersson, D.I. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* **2000**, *287*, 1479–1482. [PubMed]

48. Bellec, L.; Clerissi, C.; Edern, R.; Foulon, E.; Simon, N.; Grimsley, N.; Desdevises, Y. Cophylogenetic interactions between marine viruses and eukaryotic picophytoplankton. *BMC Evol. Biol.* **2014**, *14*, 59. [CrossRef] [PubMed]

49. Luria, S.; Delbrück, M. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* **1943**, *28*, 491–511. [PubMed]

50. Karafistan, A.; Martin, J.M.; Rixen, M.; Beckers, J.M. Space and time distributions of phosphate in the Mediterranean Sea. *Deep. Sea Res. I* **2002**, *49*, 67–82. [CrossRef]

51. Jessup, C.M.; Bohannan, B.J.M. The shape of an ecological trade-off varies with environment. *Ecol. Lett.* **2008**, *11*, 947–959. [CrossRef] [PubMed]

52. Bohannan, B.J.M.; Travisano, M.; Lenski, R.E. Epistatic Interactions Can Lower the Cost of Resistance to Multiple Consumers. *Evolution* **1999**, *53*, 292–295. [CrossRef]

53. Finkel, Z.V.; Beardall, J.; Flynn, K.J.; Quigg, A.; Rees, T.A.V.; Raven, J.A. Phytoplankton in a changing world: Cell size and elemental stoichiometry. *J. Plankton Res.* **2010**, *32*, 119–137. [CrossRef]

54. Peter, K.H.; Sommer, U. Interactive effect of warming, nitrogen and phosphorus limitation on phytoplankton cell size. *Ecol. Evol.* **2015**, *5*, 1011–1024. [CrossRef] [PubMed]

55. Atkinson, D.; Ciotti, B.J.; Montagnes, D.J.S. Protists decrease in size linearly with temperature: Ca. 2.5% $°C^{-1}$. *Proc. Biol. Sci.* **2003**, *270*, 2605–2611. [CrossRef] [PubMed]

56. Morán, X.A.G.; López-Urrutia, Á.; Calvo-Díaz, A.; Li, W.K.W. Increasing importance of small phytoplankton in a warmer ocean. *Glob. Chang. Biol.* **2010**, *16*, 1137–1144. [CrossRef]

57. Geider, R.; Platt, T.; Raven, J. Size dependence of growth and photosynthesis in diatoms: A synthesis. *Mar. Ecol. Ser.* **1986**, *30*, 93–104. [CrossRef]

58. Šupraha, L.; Gerecht, A.C.; Probert, I.; Henderiks, J. Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation. *Sci. Rep.* **2015**, *5*, 16499. [CrossRef] [PubMed]

59. Ryther, J.; Menzel, D. Light adaptation by marine phytoplankton. *Limnol. Oceanogr.* **1959**, *4*, 492–497. [CrossRef]

60. Wozniak, B.; Hapter, R.; Dera, J. Light curves of marine plankton photosynthesis in the Baltic. *Oceanologia* **1989**, *29*, 61–78.

61. Renk, H.; Ochocki, S. Photosynthetic rate and light curves of phytoplankton in the southern Baltic. *Oceanologia* **1998**, *40*, 331–344.

62. Riemann, B.; Simonsen, P.; Stensgaard, L. The carbon and chlorophyll content of phytoplankton from various nutrient regimes. *J. Plankton Res.* **1989**, *11*, 1037–1045. [CrossRef]

63. McLachlan, J. The effect of salinity on growth and chlorophyll content in representative classes of unicellular marine algae. *Can. J. Microbiol.* **1961**, *7*, 399–406. [CrossRef]

64. Sigaud, T.C.S.; Aidar, E. Salinity and temperature effects on the growth and chlorophyll-a content of some planktonic aigae. *Bol. Inst. Oceanogr.* **1993**, *41*, 95–103. [CrossRef]

65. Brockhurst, M.A.; Rainey, P.B.; Buckling, A. The effect of spatial heterogeneity and parasites on the evolution of host diversity. *Proc. Biol. Sci.* **2004**, *271*, 107–111. [CrossRef] [PubMed]