Intra-specific Variation, Selection, and Climate Change in the Seas

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Abstract

Marine ecosystems will be impacted by climate change over the next century, where increased temperature and ocean acidification are already changing the physical and chemical nature of the seas. How the marine biota will respond to these impacts is unclear, in particular we lack understanding of organisms expected evolutionary responses to climate change. Microbes (i.e. bacteria and protists) are particularly important components of marine ecosystems, due to their roles in marine food webs, and their ability to influence climate via biogeochemical cycles.

The majority of research into biological impacts of climate change is performed using short term perturbation experiments, where organisms adapted to present conditions are cultured in conditions predicted to occur up to 90 years in the future. Eco-physiological and/or life-history traits are then used to infer species responses, and in some cases fitness. This thesis suggests why this could be a misleading approach, and places emphasis on the importance of intra-specific variation in adaptation to climate change, using a series of experiments in the heterotrophic dinoflagellate *Oxyrrhis marina*.

I quantify variation in eco-physiological responses to elevated temperature and pCO_2, among geographic isolates of *Oxyrrhis*, develop a novel molecular method to quantify strain frequencies in mixed populations, and explicitly test the relationship between eco-physiology and competitive fitness. I reveal substantial variation in eco-physiological responses, and show that often this is not indicative of fitness. I then investigate frequency- and density-dependence of selection, and quantify how selection responds to environmental change. I conclude that the importance of intra-specific variation should never be overlooked, and that the best way to quantify selection from this variation is looking for relative changes in selection rates between isolates under experimental climate change conditions.

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Author's declaration

I declare that the work presented in this thesis is my own original research and that none of the work in this thesis has been previously submitted for award at this or any other institution.

Chapter 1

General Introduction

1.0 Introduction

Climate change could have potentially serious consequences for a wide range of marine organisms during the 21st century (Guinotte and Fabry 2008; Orr et al. 2005). In marine ecosystems, in addition to temperature increases surface waters are becoming acidified, a process known as ocean acidification (OA). While most biological OA research efforts have been directed towards calcifying organisms, such as corals, coccolithophorids, pteropods and foraminifera (Doney et al. 2009), and phytoplankton (Collins et al. 2014), changes in acidity are likely to impact on non-calcifying organisms, yet these potential consequences are poorly-understood. Joint et al. (2011) hypothesised that populations of marine microbes, such as protists and bacteria, may be able to tolerate anticipated levels of acidification due to high local variability and heterogeneous distribution of pH in the oceans and also because of the high evolutionary potential of microbial populations: Joint and co-workers' hypothesis largely remains untested. Understanding the response of marine microbes (i.e bacteria and protists) is critical as they form the base of marine food webs (Sherr and Sherr 1988), are responsible for the vast majority of marine primary production (Falkowski et al. 1998), and are heavily involved in biogeochemical process(Arrigo 2005), not least the carbon cycle (Longhurst 1991). Understanding how natural ecosystems, communities, species and populations will respond to climate change is crucial to mitigating anthropogenic environmental impacts and remains a major ecological and evolutionary challenge (Hoffmann and Sgro 2011).

In this thesis I use a model protist taxon, the *Oxyrrhis marina* species complex (Montagnes et al. 2010), to test fundamental ecological and evolutionary theory and address environmental questions about how natural populations may respond in a changing ocean. *Oxyrrhis* is a heterotrophic dinoflagellate that is common in tide pools across the globe, is easily isolated and cultured. *Oxyrrhis* has a high level of both functional and genetic variation (Lowe et al. 2005; Lowe et al. 2010c; Lowe et al. 2010d), with known variability in growth responses to temperature and salinity between strains (Lowe *et al.* unpublished). I use a collection of strains from European seas (see map, Fig.1.1) to test the degree of intra-specific diversity, across a defined seascape, in responses to simulated climate change. Importantly, *Oxyrrhis* is neither photoautotrophic nor calcifying, and thus represents an example of a microbial species that neither relies on inorganic carbon as a nutrient, nor has any specific or obvious physiological mechanism which could invoke a high cost under climatically relevant levels of OA. Previous work on this system has described the genetic structure of *Oxyrrhis* across the European seascape, and has shown that several distinct phylogenetic clusters form between the Atlantic Ocean and within the Mediterranean Sea (Lowe et al. 2012), probably in fact representing two species, *O. marina* and *O. maritima,* in the Atlantic and Mediterranean respectively (Lowe et al. 2010a). The focus of this thesis is upon Atlantic isolates (i.e. *Oxyrrhis marina*), though some data are presented for *O. maritima* but for the sake of brevity it should be noted that I use *O. marina* synonymously for both species within the genus *Oxyrrhis*.

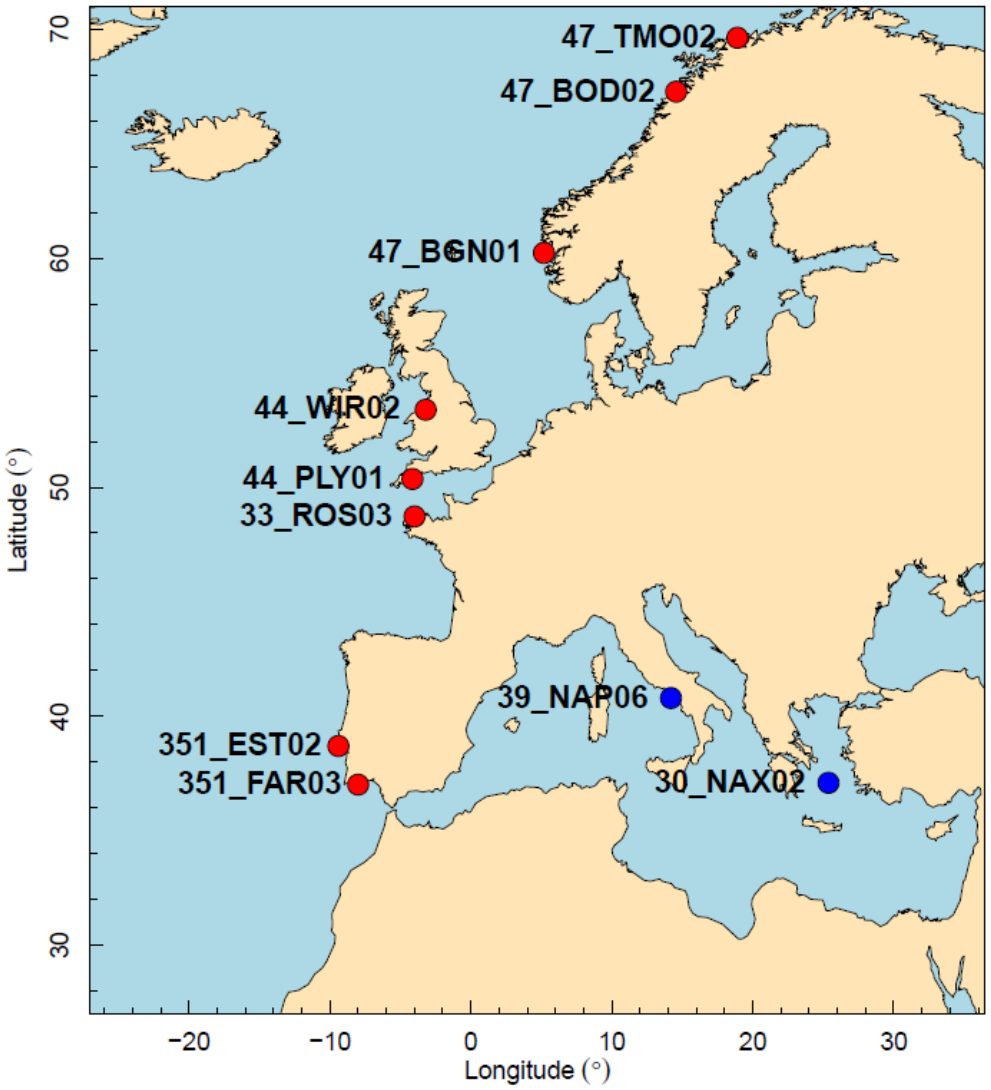

Fig.1.1. Map of Europe indicating where strains of *Oxyrrhis* used in this thesis were originally isolated. Strain names are given in text to the right of circles. Red circles indicate *O. marina,* blue circles are *O. maritima* (Lowe et al. 2010a).

1.1. Ocean acidification

Ocean acidification (OA), also known as 'the other CO_2 problem' (Doney et al. 2009), is a consequence of increased dissolved CO_2 (pCO_2) in oceanic waters. Increased pCO_2 causes a greater concentration of carbonic acid (H_2CO_3), which in turn alters the ratio of bicarbonate (HCO_3^{1-}) to carbonate (CO_3^{2-}) ions, causing an increase in H^+ ions and therefore an increase

in acidity. On average, the ocean has a pH of 8.2, but projected carbon emission scenarios predict a drop of between 0.2 to 0.7 pH units by the year 2100 (Caldeira and Wickett 2003). Although the biogeochemical understanding of OA is well developed, there remain major uncertainties, particularly because future carbon emissions are unknown. Despite uncertainties about the extent of OA, most work agrees that a global average drop in pH is inevitable.

Biological responses to OA however are far more uncertain. Crucially, present work that has examined biological responses to OA have provided conflicting results, with some species severely inhibited by acidification, while other species appear to be unaffected or display increased physiological performance (Liu et al. 2010). Indeed, a recent meta-analysis on known biological responses found negative yet variable effects of OA on marine organisms (Kroeker et al. 2010). Rates of calcification generally decrease, and, in general, calcifying organisms grow less-well under higher pCO_2, but other organisms often show no signs of inhibition and, indeed, most autotrophs increase photosynthesis and production under high pCO_2, at least in the short term (Collins and Bell 2004; Hare et al. 2007). For the autotrophic coccolithophorid, *Emiliana huxleyi*, experimenters have found a high amount of variation in the responses of calcification, growth and photosynthesis (Ridgwell et al. 2009). Whilst most research is directed towards understanding general responses of marine organisms, the high level of variation itself could be a good indicator of the potential for evolutionary adaptation (Bell and Collins 2008), as standing genetic variation is predicted to increase the extent to which populations can adapt to novel environments (Barrett and Schluter 2008).

Almost all of the information for these analyses has come from short-term perturbation experiments, typically with a short acclimation period followed by experiments spanning one or few generations. More recent research has investigated the long-term effects of OA in

multigenerational experiments. The first evolutionary studies examined adaptation to high pCO_2 in the green alga *Chlamydomonas reinhardtii* (Collins 2011; Collins and Bell 2004; Collins and de Meaux 2009) and have been followed by several studies looking at adaptation in a greater range of marine phytoplankton (Lohbeck et al. 2013; Lohbeck et al. 2012; Muller et al. 2010). These studies have provided deep evolutionary insights into adaptation in response to OA, and have shown for instance that phytoplankton are capable of an adaptive response (Lohbeck et al. 2012) but that in doing so they may lose functions considered important in the present day environment (Collins and Bell 2004), via reductive genomic evolution.

Muller et al. (2010) actually came under criticism from Collins (2010) for not doing enough to detect evolution in their experimental populations, for not measuring fitness parameters or competing evolved and control populations with each other under both conditions. This reflects the wide gap between biological oceanographers and evolutionary biologists in trying to understand how species will respond to OA. It is true that many marine biologists and biological oceanographers do not consider studying evolution in real time a realistic option, and within the various national and international ocean acidification research projects there is a lack of evolutionary studies (EPOCA (www.epoca-project.eu), BIOACID (www.bioacid.de), UKOARP (www.oceanacidification.org.uk)). Most research is conducted on organisms that are adapted to the current environment, by putting them in a heavily acidified environment, to simulate the expected environment in 90 years or more, and changes in certain parameters such as growth, survival and reproduction are measured (Dixson et al. 2010; Widdicombe et al. 2009; Widdicombe and Needham 2007; Zippay and Hofmann 2010). Not surprisingly the results often show a decrease in "performance" of some kind or another, but do these results really mean anything? The appropriate response to examine is the effects of change over evolutionary timescales, and specifically to measure

selection and changes in selection, of intra-specific variants or genotypes, that may have arisen in laboratory selection experiments having fixed beneficial mutations, or that existed in nature prior to environmental change as an estimate of pre-adaptation .

It is understandable, especially for organisms with generation times on the order of years, that studying experimental evolution may not seem appropriate, and short term studies have been useful in showing that acidification might be a problem. There is also an indication that negative responses become weaker when scaled up to apply to whole ecosystems (Leuzinger et al. 2011), this combined with a lack of evolutionary understanding means that predictions may be inappropriate. To truly assess the biological impacts of OA, and other environmental factors more generally, one must quantify whether organisms need to adapt, whether they can adapt and, if so, the mechanisms behind adaptation. Fundamentally speaking, evolutionary processes must be better integrated into assessments of species responses to climate change, and wider understanding of the range of process involved in adaptation are required in order to make this applicable to natural systems.

1.2. Adaptation to climate change

There has been a considerable recent research effort to understand how species, populations and communities will respond to climate change (Hoffmann and Sgro 2011; Lavergne et al. 2010). The majority of this research has been conducted using field data from terrestrial animal and plant systems (Cramer et al. 2001; McMahon et al. 2011; Thomas et al. 2004), although there is considerable experimental and theoretical research using other systems (Moloney et al. 2011; Perry et al. 2005). Current understanding of how populations respond to climate change may be broadly divided into three main components, with most research focussing on a single component only.

Firstly, phenotypic plasticity permits a rapid response to environmental change within an individual's lifespan (Ghalambor et al. 2007). Populations exposed to environmental parameters, such as temperature, light, food availability, and salinity that change over short temporal and small spatial scales, are presumably adapted to survive these fluctuating conditions (Hofmann and Todgham 2010). Climate change is concerned with gradual changes over longer timescales, and so many populations will have mechanisms to produce optimum phenotypes, but only within the limits of plastic variation. Many studies have found that changes in phenology are already occurring through phenotypic plasticity (Charmantier et al. 2008; Nussey et al. 2005; Reale et al. 2003) and that these changes can have knock on effects for survival and other phenotypes (Ozgul et al. 2010). Theoretical work has shown that plasticity may contribute to adaptive genetic change via genetic assimilation (Lande 2009).

Second, and perhaps most significantly, species and populations will alter their geographic distributions. Indeed, numerous studies have observed a shift to higher latitudes, which has been attributed to global warming (Barry et al. 1995; Davis and Shaw 2001; Murawski 1993; Parmesan et al. 1999) as species attempt to maintain their thermal niche. This behavioural solution to changing temperatures brings with it, for example, the indirect consequences of population translocation, and altered community interactions (Perry et al. 2005).

Finally there is evolution by natural selection on standing genetic variation and/or *de novo* mutations - the classic view of evolution that is measured by genetic changes in time and space (Lande 1976). While several case studies on wild populations have uncovered genetic changes in response to climate change (Bradbury et al. 2010; Bradshaw and Holzapfel 2001; Ellison et al. 2011), much work on the evolutionary response to a changing environment has been conducted in the laboratory using a model systems approach (Bell and Gonzalez 2009;

Bettencourt et al. 2002; Collins and Bell 2004; McColl et al. 1996). Many experiments, especially in microbial systems, begin with a single clone, or at least a fairly homogenous population, and quantify selection acting on *de novo* mutations. Such experiments have shown increased pCO_2 to cause selection on genes involved in pH homeostasis and calcification in sea urchins (Pespeni et al. 2013), possible loss of function mutations in carbon concentrating mechanism genes for freshwater phytoplankton (Collins and Bell 2004), and have shown species of marine phytoplankton to be capable of adaptation with no obvious mechanism (Benner et al. 2013), and via changes in gene expression (Lohbeck et al. 2014). Insights into OA adaptation from experimental evolution are still in their infancy but this powerful technique is rapidly expanding our knowledge of how species may respond to this environmental change.

Despite an accumulation of observations of these responses, a mechanistic and integrated theory combining these observations is still lacking (Lavergne et al. 2010; Matthews et al. 2011). We do not know the relative contributions of each response to selection, or under which circumstances each response plays a greater role in adaptation and ultimately species' persistence. Furthermore, we do know that biological interactions often drive rapid evolution (Paterson et al. 2010; Turcotte et al. 2011) and that to an individual or a population, the biotic environment can be equal to or greater than the abiotic environment in importance. Given that we are already seeing extinctions and range shifts, it is likely that these highly complex networks will be significantly altered (Guimaraes et al. 2011), and that selection to temperature or acidification could be insignificant when compared to selection to intra and inter-specific interactions.

1.3. Intra-specific variation

Natural populations are often genetically diverse (Kashtan et al. 2014; Lowe et al. 2005), and evolution from standing genetic variation can occur much quicker than that acting on new mutations (Hermisson and Pennings 2005). Selection from standing genetic variation (Barrett and Schluter 2008) is less completely understood when compared with selection on *de novo* mutants and presents some challenges in terms of experimental tractability. Natural selection relies on genetic variation upon which to act, and increased standing variation provides a wider range of genotypes which could be better pre-adapted to novel conditions, in addition to increasing the likelihood of beneficial mutations arising in a complimentary genetic background. Standing variation is one of the key factors increasing the probability of evolutionary rescue (Bell and Collins 2008). In a climate change context standing variation could be a particularly important source of evolutionary innovation, as environmental change in the ocean represents a gradual shift in mean temperature and pCO_2 levels, well within the natural range of conditions experienced over relatively short spatial and even temporal scales. Hence, populations locally adapted to warmer environments, for instance, could represent a reservoir of genes that may facilitate adaptation on the species level, to increased temperatures. The importance of intra-specific variation has been recognised for some time, however many studies that examine this variation rarely attempt to quantify selection from standing variation, and instead infer fitness via eco-physiological rates, such as growth, to predict adaptation.

The focus of this thesis is on intra-specific variation, and its role in evolutionary adaptation in response to increased temperature and pCO_2. In Chapter 2 I quantify intra-specific variation in eco-physiological responses to these climate change conditions, and find that whilst substantial variation exists, it cannot be explained by adaptation to prevailing environmental

conditions. Next, in Chapter 3 I present a method to allow determination of strain specific frequencies in mixed populations of *Oxyrrhis,* and use it to show that growth rates in isolation are poor predictors of competitive fitness. In Chapter 4 I examine the relative roles of frequency- and density- dependence of selection for *Oxyrrhis*, where I show that frequency dependence is stronger at higher population densities. Finally, in Chapter 5 I perform competition experiments between many pairs of strains, under elevated temperature and pCO_2 conditions to show that direct estimation of selection is more predictive of local adaptation than eco-physiological rates, and that strains of *Oxyrrhis* from environments of higher absolute pCO_2 may be better adapted to a future acidified ocean.

Chapter 2

Intraspecific variation in the eco-physiological response to rising temperature and pCO_2 is not explained by strain specific environmental background

2.0 Introduction

Global climate change is expected to have a major global impact, particularly upon marine ecosystems (Hoegh-Guldberg and Bruno 2010) where temperature increases will be accompanied by acidification. Over the next century, marine habitats are expected to warm, on average, by around 1.8-4.0°C (Collins et al. 2013) and experience an increase in acidity of some 0.07-0.33 pH units (Bopp et al. 2013), largely following the global rise in partial pressure of atmospheric carbon dioxide (pCO_2) from pre-industrial levels of ~280 µatm to projected levels of 490-1,370 µatm (van Vuuren et al. 2011). Understanding the biological responses to these environment challenges is crucial for biodiversity conservation *per se* (Hannah et al. 2000), and because marine organisms contribute to a host of global biogeochemical processes (Arrigo 2005) and, in particular, the carbon cycle (Longhurst 1991).

Much of our understanding of organismal responses to environmental stressors, such as acidification and temperature increases, come from laboratory experiments that measure rate processes such as growth, photosynthesis, and calcification (Gao and Zheng 2009; Iglesias-Rodriguez et al. 2008; Kaniewska et al. 2012), or life history traits such as size, fecundity, and mortality (Cripps et al. 2014; Ellis et al. 2009; Gazeau et al. 2011). However, most

studies infer a species' response to changing climate with data from one or few isolates of the target species (Doo et al. 2011; Munday et al. 2009) or even infer a major functional group's response with just one isolate of a few species (Hutchins et al. 2013). Of course, eco-physiological responses can vary substantially within species (Kremp et al. 2012; Ridgwell et al. 2009; Schaum et al. 2012), for example due to experimental procedures or genetic variation. Given that there may be a large amount of cryptic genetic variation within species (Lowe et al. 2012; Lowe et al. 2010c), inferring the response(s) of one or few isolates neglects the potential importance of genetic variation as fuel to adaptive evolutionary change (Barrett and Schluter 2008) and will likely lead to incorrect conclusions about how species will respond to environment change.

Standing genetic variation has potential to contribute to evolutionary rescue (Bell 2013): increased genetic diversity provides a wider range of solutions for natural selection to act upon, by both reducing reliance on *de novo* mutations and providing more backgrounds against which beneficial mutations can arise. Standing genetic variation is thus likely to be an important component of population evolvability (Bell and Collins 2008). Gene flow between and recombination may then re-assort advantageous alleles to facilitate adaptation. Thus, assessing intra-specific variation in natural populations is an important component of understanding biotic responses to climate change. A potential limitation with studies that assess the role of diversity *per se* is that they fail to recognise that strains from different populations may have adapted to different environments. For example, strains that inhabit, and presumably are adapted to, warmer areas of a species' distribution are likely to have a different response to rises in temperatures than strains from a colder area. Thus, studies that assess the role of diversity should take into account the population origin as well as the amount of diversity in itself.

Marine microbes account for ~50% of global primary production (Longhurst et al. 1995) and the majority of the subsequent transfer or carbon to higher trophic levels (Pernthaler 2005). Due to their importance at the base of the marine food chain, their ability to influence climate, and because they underpin ecosystem function, making accurate predictions about how microbes will respond to climate change is of paramount importance. Emerging data indicate both detrimental and positive effects of rising levels of dissolved CO_2 and the concomitant decrease in pH (i.e. ocean acidification - OA) in marine systems; for example, greater dissolved CO_2 is expected to decrease rates of calcification (Beaufort et al. 2011), but may increase phototrophic production (Low-Decarie et al. 2011) in areas where CO_2 is a limiting nutrient for certain phytoplankton (Low-Decarie et al. 2014). In both cases the responses do not appear to be uniform across taxa, indicating that there will be 'winners and losers' (Collins et al. 2014; Ries et al. 2009). Moreover, such research has emphasised photoautotrophic and calcifying microbes (e.g. coccolithophores) where there is an obvious physiological response associated with rising acidity. The effects of OA on non-photosynthesising, non- calcifying marine microbes have received less attention and predictions of their

Table 2.1. Sampling information for strains of *Oxyrrhis* used in this thesis.

Strain ID	Sampling location	Country	Latitude (N)	Longitude (W)	Date Isolated
30_NAX02	Naxos	Greece	37° 5' 41.28"	25° 22', 25.68"	24/11/2008
39_NAP06	Naples	Italy	40° 47' 49.94"	14° 11' 53.88"	08/08/2008
351_FAR03	Faro	Portugal	37° 1' 1.1"	-7° 59' 35.5"	22/01/2008
351_EST02	Estoril	Portugal	38° 42' 6.84"	-9° 23' 34.8"	09/07/2009
33_ROS03	Roscoff	France	48° 43' 38.56"	-3° 59' 21.51"	17/05/2009
44_PLY01	Plymouth	UK	50° 21' 47.52"	-4° 8' 20.76"	25/04/2008
44_WIR02	Wirral	UK	53° 23' 13.59"	-3° 12' 0.51"	01/03/2009
47_BGN01	Bergen	Norway	60° 14'8.00"	5° 11'8.00"	22/07/2009
47_BOD02	Bodo	Norway	67° 16' 33.24"	14° 34' 13.80"	25/06/2011
47_TMO01	Tromso	Norway	69° 37' 48.00"	18° 54' 36"	15/08/2011

responses tend to be based on the assumption that increased acidity may be stressful in itself. In laboratory experiments increased temperature tends to increase microbial growth rates (Epply 1972) and decrease cell size (Atkinson et al. 2003), yet again there is considerable variation within and between species (Kaeriyama et al. 2011; Weisse and Montagnes 1998).

Here, I use experiments on the marine flagellate *Oxyrrhis marina* to quantify intra-specific variation in eco-physiological (growth rate and cell size) responses to increased levels of pCO_2 and temperature that are in line with the IPCC forecasts (Collins et al. 2013). *Oxyrrhis marina* is a heterotrophic, non-calcifying flagellate that often is abundant in marine intertidal habitats and estuaries worldwide (Watts et al. 2010). It is widely-used as an ecological model organism to represent marine microzooplankton and protistan biogeography. I examine the extent of variation in responses among nine isolates of *O. marina* collected from European coastal marine environments that span a latitudinal range of more than 30° (3,000 km). I show that the extensive eco-physiological variation in strain response to temperature and pCO_2 cannot be simply related to the environmental background from which strains were derived.

2.1 Methods and Materials

Experimental strains and culturing

I examined nine strains of the heterotrophic flagellate *Oxyrrhis marina* Dujardin 1895, isolated from a range of European coastal environments, with two strains originating from the Mediterranean Sea and seven strains isolated from the North East Atlantic Ocean: 30_NAX02 (Naxos, Greece), 39_NAP01 (Naples, Italy), 351_EST02 (Estoril, Portugal), 351_FAR01 (Faro, Portugal), 33_ROS03 (Roscoff, France), 44_PLY01 (Plymouth, UK),

44_WIR02 (Wirral, UK), 47_BGN01 (Bergen, Norway), 47_BOD01 (Bodo, Norway), and 47_TMO01 (Tromso, Norway) (see Table 2.1, Fig. 1.1). These strains were selected as they represent a wide range of environmental backgrounds, for both temperature and pCO_2.

After the initial isolation from environmental water samples, stock cultures were maintained in the laboratory at 16°C, under a light:dark cycle of 14:10, at a light intensity of ~80 µmol photons m^{-2}, in a medium of 32 PSU sterile filtered artificial seawater (SASW) enriched with f/2 (Sigma Aldrich, UK), and fed on *Dunaliella primolecta* at an initial cell density of ~3×10^5 cells ml^{-1}. Stock cultures were maintained by sub-culturing once per month. Prior to the growth experiments, strains were sub-cultured in the dark and fed on heat-killed *Escherichia coli* (Lowe et al. 2010b) to deplete *D. primolecta*: these are the 'pre-experimental' cultures.

Growth experiments

Growth experiments were performed to quantify the effects of increased temperature and pCO_2 on growth rate (r) and cell size. Acclimation cultures were grown in 500 mL Duran flasks containing ~300 mL of media that was inoculated with 1,000-2,500 cells ml^{-1} of *O. marina*, 100 µg mL^{-1} penicillin/streptomycin, 50 µg mL^{-1} gentomycin (Lonza, UK), and heat-killed *E. coli* (1.25-2.5×10^6 cells ml^{-1}). Acclimation cultures were grown at 16°C and 20°C and aerated with pre-mixed CO_2/Air mixtures (BOC Special Products, UK) with the desired CO_2 concentrations for 72 hours prior to the start of the experiments. There was one acclimation culture per treatment group (six in total). Treatment groups had pCO_2 values of approximately 380, 750, and 1,100 µatm and temperatures of 16°C and 20°C in a fully factorial design. Gas concentrations differed between experiments on different strains according to the batch of pre-mixed CO_2/Air (supplied by BOC Special Products, UK), which had different partial pressures of CO_2.

Experimental incubations were inoculated in triplicate with 750 cells mL^{-1} from the acclimation cultures into 200 mL of fresh SASW media (32 psu) and fresh heat killed *E. coli* (no antibiotics were used during the experiments). Ten mL samples were taken at 0, 24 and 48 hours from the experimental microcosms and were fixed with 2% (v/v) Lugol's iodine directly into 10 mL Utermohl chambers. Samples were allowed to settle for around 20 hours before being imaged using a Zeiss Axiovert A1 inverted microscope (Carl Zeiss, Germany) according to the methods described in Appendix 1.

Strain environmental background

Data about the environments from which the strains were isolated were derived from the Surface Ocean CO_2 Atlas (SOCAT) (Bakker et al. 2014) using the "Cruise Data Viewer" online tool. The SOCAT database is an amalgamation of various oceanographic datasets for surface ocean measurements of fCO_2 derived from direct measurements of CO_2 in either seawater equilibrated air, or from seawater itself (Pfeil et al. 2013). This data set has been rigorously quality controlled and provides a consistent set of measurements for fCO_2 across the world's oceans. All localities contain a sea surface temperature (SST) measurement, recorded at the same time as the CO_2 measurements. Data was collected from the database to represent all Atlantic strains used in this study (Fig.2.1.), however coverage of The Mediterranean Sea in the SOCAT database was not extensive enough to provide data for the Mediterranean strains.

Data for the North East Atlantic Ocean (-25°W, 30°E, 30°S, 75°N), for 2005 until 2011, were extracted and then subdivided by location to provide measurements associated with the sample location of each Atlantic strain of *Oxyrrhis* (Table 2.1). For each strain I thus had associated data for mean annual SST, mean annual fCO_2, mean spring SST, mean spring fCO_2, intra-annual SST variation, intra-annual fCO_2 variation, sampling month mean SST,

and sampling month mean fCO_2; annual means thus included data recorded on any date, while spring was defined as the months of April and May, and sampling month was defined as the date in the year a strain was sampled (Table 2.1) ±15 days. Intra-annual variation was defined as the standard deviation of measurements from all data sampled throughout the year.

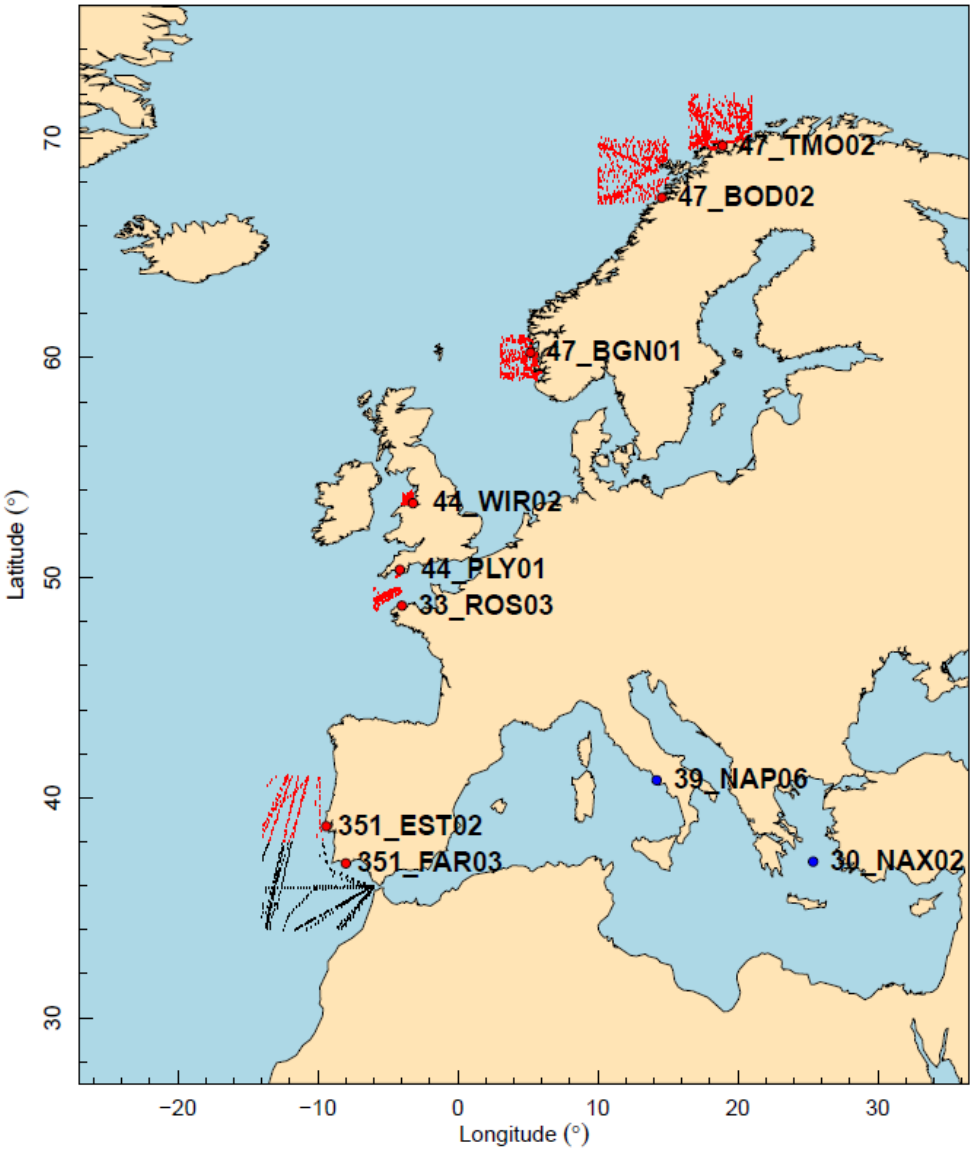

Fig.2.1. Map of Europe indicating geographical distribution of samples from the SOCAT database used to infer environmental parameters for individual strains. Data for 351_FAR03 are in black, due to close proximity with 351_EST02.

Data analyses

Images were analysed using ImageJ (NIH, USA) with an automated macro (Appendix 1) that measured cell density and average cell size within a sample for five images per sample. Growth rates (day^{-1}) were calculated as slope of natural log of cell density with time, and average cell size was calculated as the mean cross sectional area (μm^2) of all cells counted in an image for the final sampling point only. Eco-physiological responses (i.e. growth rate and cell size) for each strain were analysed by two-way ANCOVA with pCO_2 as a continuous variable and temperature as a factor. A global model for both growth rate and cell size was analysed with strain as an additional factor.

To examine whether environmental background was predictive of a strain's eco-physiological response, a metric of response size was calculated for both growth rate and cell size as the slope of the measured variable against either temperature or pCO_2 with all experimental treatments combined. These are termed the "temperature/pCO_2–growth/size response", and were analysed by linear least-squares regression environmental parameters for each Atlantic strain estimated from the oceanographic data. All analyses were performed using R v.3.1.0 (R Core Development Team, 2014).

2.2 Results

Effects of temperature and pCO_2 on growth rate

The variation in temperature and pCO_2 that I examined were not lethal to *Oxyrrhis marina*, with all cultures experiencing positive growth rates over the 3 day period (Fig. 2.2.). I observed strain-specific growth rate responses to both temperature (ANCOVA, strain*temp interaction, $F_{8,135}=2.545$, $P<0.05$) and pCO_2 (ANCOVA, strain*pCO_2

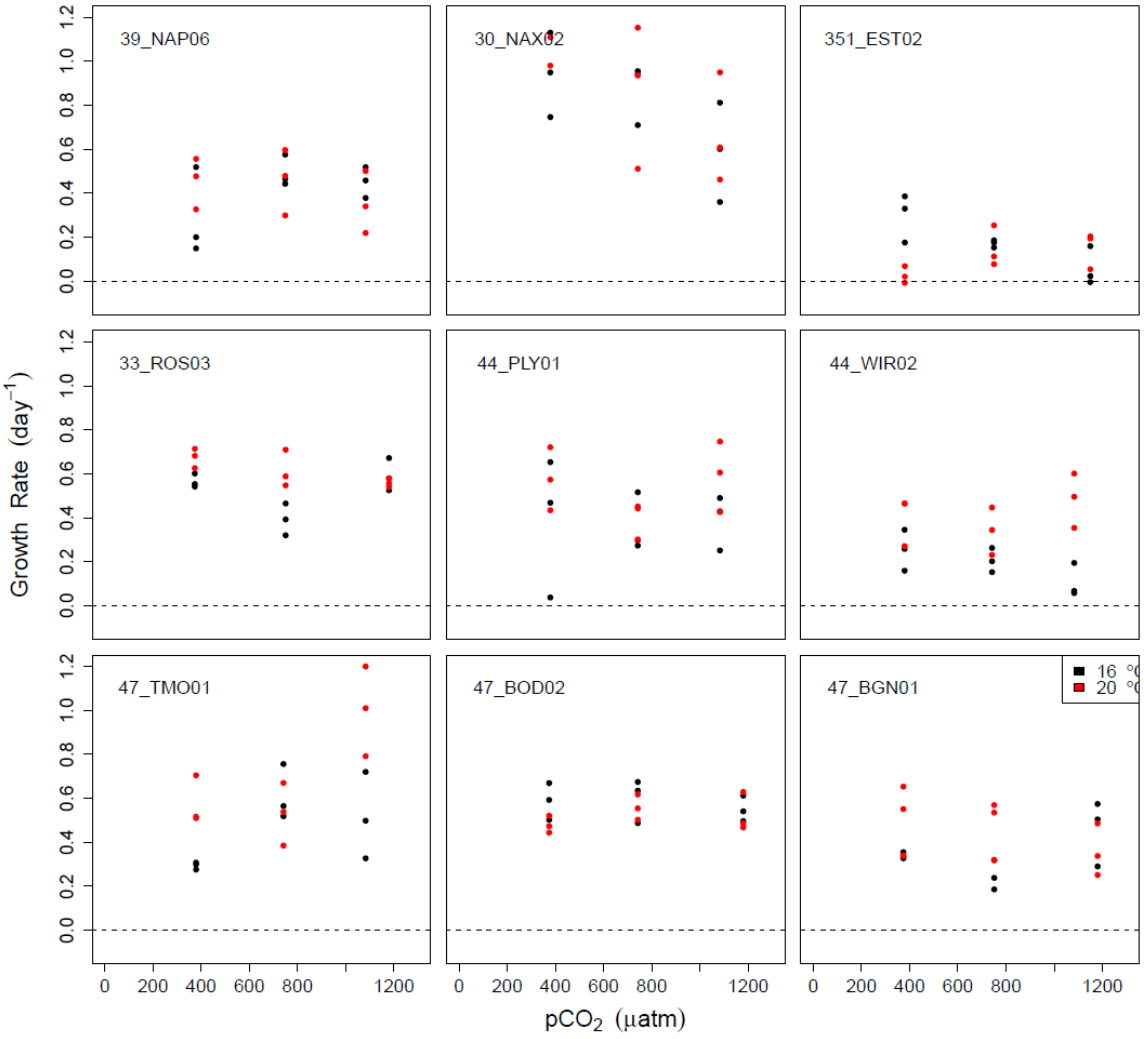

Fig.2.2. Growth rates of nine strains of *Oxyrrhis* in response to pCO_2 at 16 °C (Black) and 20 °C (Red). Strains are indicated in the top left of each panel.

interaction, $F_{8,135}$=5.046, P<0.001) but no systematic effect of temperature on pCO_2 responses (ANCOVA, temp*pCO_2 interaction, $F_{1,135}$=0.008, P=0.93). To quantify the pattern of this variation further, I analysed each strain's growth rate responses separately and found substantial variation to the growth rates between strains of *Oxyrrhis* (Fig. 2.3). Mean clonal growth rates across all environmental treatments varied from 0.14±0.03 day^{-1} (351_EST02) up to 0.85±0.06 day^{-1} (30_NAX02). Among strains there was high variation in the responses of growth to both temperature and pCO_2 (Fig. 2.3). Hence, there was no clear growth response of four strains (39_NAP06, 44_PLY01, 47_BOD02, and 47_BGN01) to variation in

either pCO_2 or temperature (Table 2.2, Fig. 2.3). Growth of three strains (33_ROS03, 44_WIR02, and 47_TMO01) were affected by temperature, exhibiting a significant increase at 20°C (Table 2.2). Two strains were impacted by altered levels of CO_2, with 30_NAX02 and 47_TMO01 growing significantly slower and faster at higher pCO_2 levels respectively. For the strain 351_EST02 I found a significant interaction between pCO_2 and temperature on growth rate (Table 2.2, Fig. 2.2).

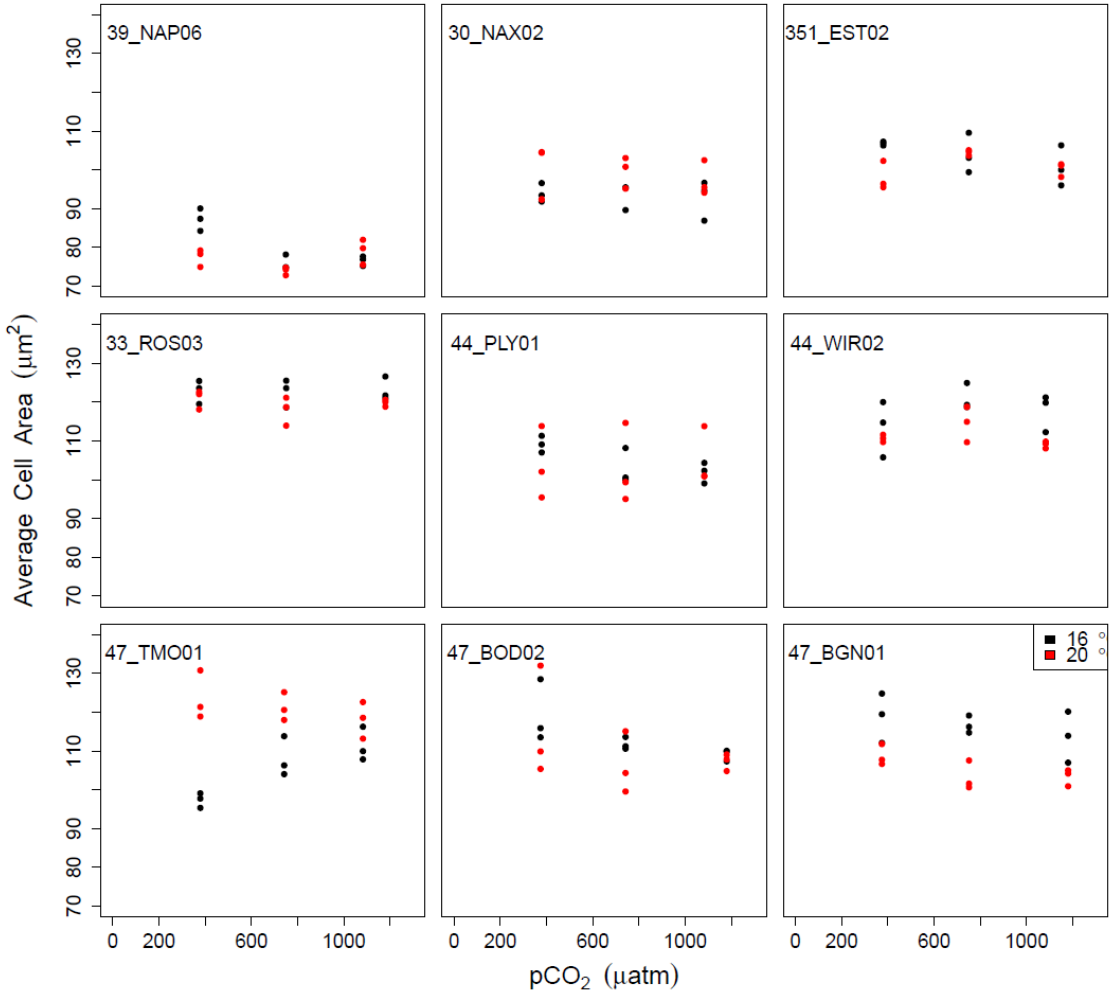

Fig.2.3. Average cell sizes for nine strains of *Oxyrrhis* in response to pCO_2 at 16 °C (Black) and 20 °C (Red). Strains are indicated in the top left of each panel.

Table 2.2. ANCOVA results for growth rates in response to pCO_2 and temperature treatments. Minimal global model is presented, models with interactions, where significant, or containing both treatments are presented for individual strains.

Strain	Treatment	d.f.	sum sq	mean sq	F	P
All Strains	CO_2	1	0.0190	0.0185	0.914	0.34089
	Temp	1	0.2960	0.2957	14.572	0.00021
	Strain	8	5.7010	0.7126	35.120	< 2e-16
	Temp*Strain	8	0.4130	0.0516	2.545	0.01296
	CO_2*Strain	8	0.8190	0.1024	5.046	0.00002
	Residuals	135	2.7390	0.0203		
39_NAP06	CO_2	1	0.0035	0.0035	0.177	0.68000
	Temp	1	0.0004	0.0004	0.022	0.88400
	Residuals	15	0.3003	0.0200		
30_NAX02	CO_2	1	0.4982	0.4982	11.504	0.00402
	Temp	1	0.0385	0.0385	0.889	0.36078
	Residuals	15	0.6495	0.0433		
351_EST02	CO_2	1	0.0100	0.0100	1.722	0.21048
	Temp	1	0.0206	0.0206	3.533	0.08114
	CO_2*Temp	1	0.0969	0.0969	16.613	0.00113
	Residuals	14	0.0816	0.0058		
33_ROS03	CO_2	1	0.0048	0.0048	0.568	0.46290
	Temp	1	0.0445	0.0445	5.229	0.03720
	Residuals	15	0.1275	0.0085		
44_PLY01	CO_2	1	0.0002	0.0002	0.007	0.93620
	Temp	1	0.0920	0.0920	3.108	0.09820
	Residuals	15	0.4441	0.0296		
44_WIR02	CO_2	1	0.0033	0.0033	0.281	0.60380
	Temp	1	0.2165	0.2166	18.451	0.00064
	Residuals	15	0.1761	0.0117		
47_TMO01	CO_2	1	0.3104	0.3104	9.123	0.00861
	Temp	1	0.2354	0.2354	6.919	0.01892
	Residuals	15	0.5103	0.0340		
47_BOD02	CO_2	1	0.0000	0.0000	0.003	0.96000
	Temp	1	0.0148	0.0148	2.810	0.11400
	Residuals	15	0.0790	0.0053		
47_BGN01	CO_2	1	0.0009	0.0009	0.050	0.82500
	Temp	1	0.0461	0.0461	2.523	0.13300
	Residuals	15	0.2739	0.0183		

Table 2.3 ANCOVA results for cell size in response to pCO_2 and temperature treatments. Models presented as in Table 2.2.

Strain	Treatment	d.f.	sum sq	mean sq	F	P
All strains	CO_2	1	87.0	86.60	3.739	0.055
	Temp	1	47.0	46.90	2.022	0.157
	Strain	8	23211.0	2901.40	125.226	< 2e-16
	CO_2*Temp	1	0.0	0.20	0.009	0.926
	CO_2*Strain	8	352.0	44.00	1.901	0.065
	Temp*Strain	8	2105.0	263.10	11.356	5.02E-12
	CO_2*Temp*Strain	8	540.0	67.50	2.914	0.005
	Residuals	126	2919.0	23.20		
39_NAP06	CO_2	1	66.2	66.22	5.660	0.032
	Temp	1	42.4	42.39	3.623	0.078
	CO_2*Temp	1	113.5	113.46	9.699	0.008
	Residuals	14	163.8	11.70		
30_NAX02	CO_2	1	13.9	13.93	0.796	0.386
	Temp	1	150.5	150.52	8.600	0.010
	Residuals	15	262.5	17.50		
351_EST02	CO_2	1	11.9	11.94	0.718	0.410
	Temp	1	38.6	38.56	2.320	0.149
	Residuals	15	249.3	16.62		
33_ROS03	CO_2	1	0.4	0.41	0.053	0.820
	Temp	1	49.2	49.17	6.338	0.024
	Residuals	15	116.4	7.76		
44_PLY01	CO_2	1	26.1	26.07	0.613	0.446
	Temp	1	1.8	1.84	0.043	0.838
	Residuals	15	637.9	42.53		
44_WIR02	CO_2	1	6.2	6.20	0.270	0.611
	Temp	1	164.2	164.20	7.157	0.017
	Residuals	15	344.1	22.94		
47_TMO01	CO_2	1	54.2	54.20	2.783	0.117
	Temp	1	1072.2	1072.20	55.066	3.25E-06
	CO_2*Temp	1	288.6	288.60	14.823	0.002
	Residuals	14	272.6	19.50		
47_BOD02	CO_2	1	251.2	251.18	4.939	0.042
	Temp	1	59.4	59.42	1.168	0.297
	Residuals	15	762.8	50.85		
47_BGN01	CO_2	1	80.9	80.90	4.877	0.043
	Temp	1	573.0	573.00	34.556	3.03E-05
	Residuals	15	248.7	16.60		

Effects of temperature and pCO_2 on cell size

The effects of temperature and pCO_2 on *O. marina* cell size were complex, with the global model identifying a significant three-way interaction between strain, temperature, and pCO_2 (ANCOVA, strain*temp*pCO_2, $F_{8,126}$=2.914, P<0.01) (Table 2.3). Average cell size across treatments (on the final day of the experiments) varied between strains, with the smallest being 39_NAP09 (78.4±1.1 μm^2) and the largest being 33_ROS03 (121.2±0.7 μm^2). As with growth rate, there was much variation between strains in the response of cell size to both temperature and pCO_2 (Fig.2.3.). For example, two strains showed no significant effect of pCO_2 or temperature upon cell size (351_EST01 & 44_PLY01), while two other strains (33_NAX02 & 47_TMO01) were significantly larger when grown at a higher temperature and yet two other strains (44_WIR02 & 44_ROS02) became significantly smaller as temperature increased (Table 2.3, Fig.2.3.). One strain (47_BOD02) became smaller at elevated pCO_2 although the effect was fairly weak (Table 2.3). Two strains (39_NAP06 & 47_TMO01) showed a significant interaction between pCO_2 and temperature on cell size (Table 2.3), whereby at low pCO_2 their cell sizes varied considerably with temperature, and as pCO_2 increased this difference became much smaller, or disappeared entirely (see Fig.2.3.).

Environmental background as a predictor of response

For growth responses, in all cases except for intra-annual temperature variation, there was a qualitative, but non-significant, negative association between the response size and environmental conditions near the strain's isolation location (Fig.2.4.). Thus, the environmental conditions from where strains were derived did not predict the strain responses to both increased temperature and increased pCO_2. There were no significant relationships between the growth-temperature response and mean annual temperature, mean spring

temperature, intra-annual temperature variability, or sampling date temperature (Table 2.4.). Similarly, I identified no significant predictors for the cell size-temperature response of strains: there were no significant relationships between the growth-pCO_2 response nor the size-pCO_2 response with mean annual fCO_2, mean spring fCO_2, intra-annual fCO_2 variability, or sampling date fCO_2 (Table 2.4.).

Table 2.4. Regression statistics for relationships between temperature- and pCO_2-growth and size responses of North Atlantic strains of *Oxyrrhis* and estimated environmental parameters from which strains were isolated.

Response	Environmental parameter	Slope	Intercept	F	d.f.	R2	P
Temperature-growth	Spring SST	-0.003	0.055	0.882	1,5	0.150	0.391
	Annual SST	-0.002	0.059	0.885	1,5	0.150	0.390
	Annual SST variability	0.012	-0.006	0.673	1,5	0.119	0.450
	Sampling date SST	-0.005	0.079	0.357	1,5	0.416	0.118
pCO_2-growth	Spring pCO_2	-0.0000007	0.00026	0.052	1,5	0.010	0.828
	Annual pCO_2	-0.0000026	0.00094	0.966	1,5	0.162	0.371
	Annual pCO_2 variability	-0.0000035	0.00017	0.505	1,5	0.092	0.509
	Sampling date pCO_2	-0.0000005	0.00020	0.090	1,5	0.018	0.776
Temperature-size	Spring SST	-0.09	0.70	0.799	1,5	0.138	0.412
	Annual SST	-0.83	0.80	0.771	1,5	0.134	0.420
	Annual SST variability	-0.50	1.07	1.630	1,5	0.246	0.258
	Sampling date SST	-0.06	0.61	0.530	1,5	0.096	0.499
pCO_2-size	Spring pCO_2	0.000035	-0.0123	0.940	1,5	0.158	0.377
	Annual pCO_2	0.000033	-0.0126	0.904	1,5	0.153	0.386
	Annual pCO_2 variability	0.000025	-0.0019	0.138	1,5	0.027	0.726
	Sampling date pCO_2	0.000029	-0.0108	3.173	1,5	0.388	0.135

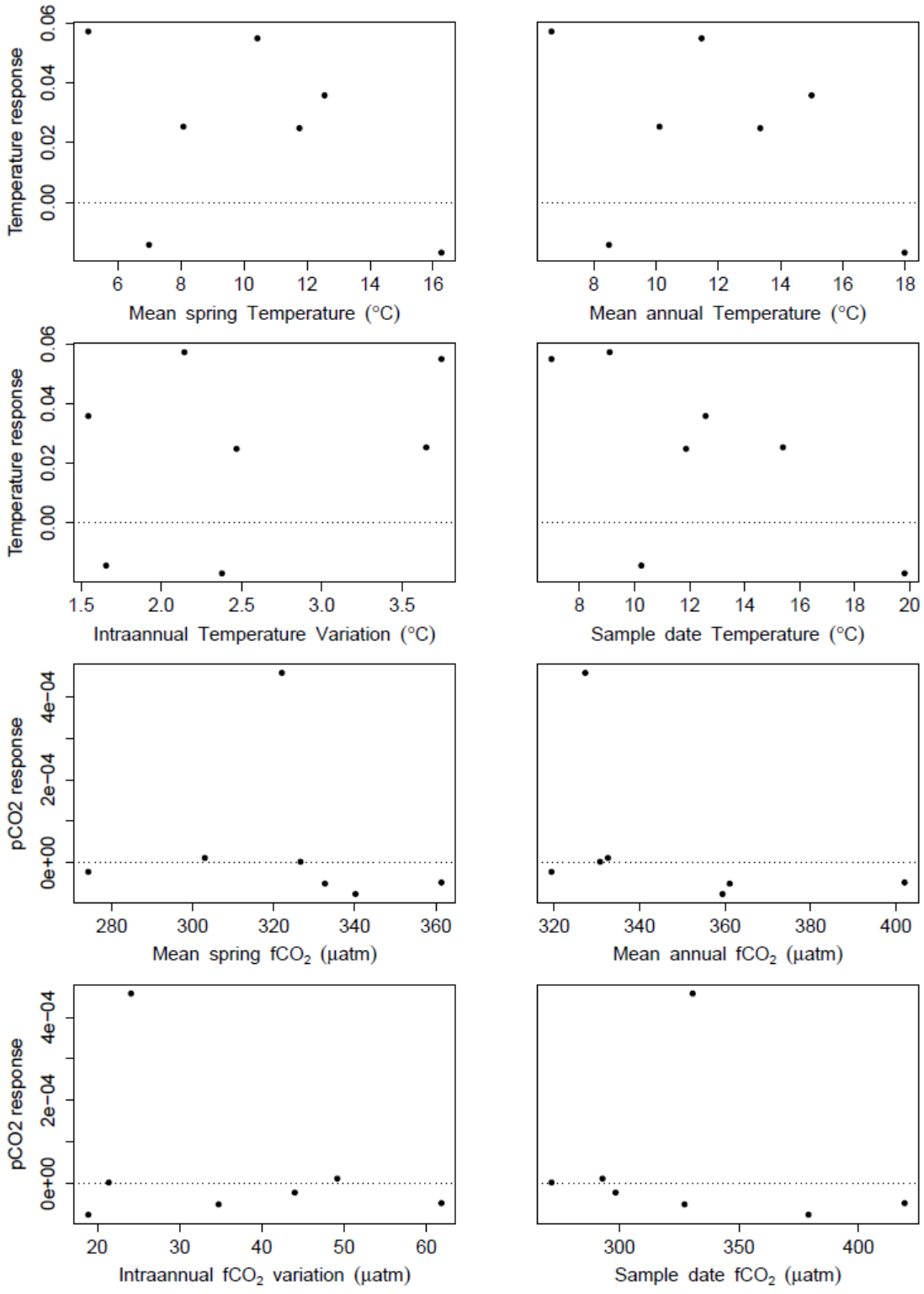

Fig.2.4. Relationship between temperature- and pCO_2-growth responses of North Atlantic strains of *Oxyrrhis* and estimated environmental parameters from which strains were isolated. Each point represents one strain.

2.3 Discussion

Experimentally derived eco-physiological responses are widely-employed to infer how populations or species may respond to global climate change impacts, such as rising temperature and levels of atmospheric carbon dioxide (Berge et al. 2010; Brading et al. 2011; Crawfurd et al. 2011; Cripps et al. 2014; Dixson et al. 2010; Hutchins et al. 2013; Riebesell et al. 2000; Widdicombe and Needham 2007). My experiments highlight a number of the key issues regarding this approach by demonstrating (1) high intra-specific variation in eco-physiological response (population growth rate and cell size) to increased temperature and pCO_2 and (2) that a strain's response *per se* may have no simple predictor relating to the strain's contemporary habitat. This finding has two important implications for understanding the ecological and evolutionary responses of species to climate change: firstly, that predictions inferred from one or few strains underestimate the response of a population or species as a whole. Secondly, that the presence and structure of intraspecific diversity may be one of the key predictors of how a species as a whole will adapt to a changing environment, for example by identifying hot spots of adaptive variation in natural populations.

I found a wide range of responses of growth and cell size to pCO_2 and temperature, with the responses largely being specific to individual strains and not predicted by the general environmental conditions from which the strains were derived. Changes in growth rates where they occurred were modest, especially with respect to pCO_2. Interactions between pCO_2 and temperature were rare in my data, again in contrast with the general finding of synergism as inferred from a sample of predominantly calcifying or phototrophic organisms (Harvey et al. 2013). Given that temperature and pCO_2 have only modest effects on *Oxyrrhis* eco-physiology, a lack of synergy is unsurprising and again may reflect a wide tolerance range for both environmental effects.

An increased growth rate is a common response of organisms to higher temperatures (Epply 1972; Savage et al. 2004), but, in just four out of nine cases I detected a significant temperature response in *Oxyrrhis*. Moreover, while protist cell sizes decrease linearly with increased temperature (Atkinson et al. 2003), I found both increases and decreases in cell size with temperature, as well as many strains showing no response in cell size to a 4°C rise in temperature. This diversity of responses exhibited by *O. marina* to temperature could reflect the fact that, as an inhabitant of coastal/intertidal areas, many populations naturally experience large fluctuations in temperature and pCO_2, even on a daily basis, and this species is therefore capable of tolerating a wide range of temperatures.

Responses to pCO_2 were comparatively small for both growth rate and cell size, though some strains did show a significant response. As *Oxyrrhis* is neither photoautotrophic nor calcifying there is no *a priori* reason to predict that should be impacted by changing CO_2 levels, unless the concomitant reduction in pH (i.e. ~0.2-0.3 units) is stressful in itself. Again, where significant responses were observed the effects were varied (i.e positive and negative changes in growth occurred). The implication is that *Oxyrrhis* may be adapted to deal with large fluctuations in pCO_2 in its natural environment (Duarte et al. 2013; Hofmann et al. 2011). Indeed, other coastal and intertidal organisms appear less sensitive than open ocean organisms to ocean acidification (Beaufort et al. 2011; Schaum et al. 2012). By contrast the globally distributed Coccolithophore *Emiliana huxleyi* exhibits high variation between strains in the response of growth and calcification to increased pCO_2 (Feng et al. 2008; Iglesias-Rodriguez et al. 2008; Riebesell et al. 2000) , likely through natural variation between ecotypes (Ridgwell et al. 2009). In addition, the globally distributed phytoplankton species *Ostreococcus tauri* has high levels of variation in the plasticity of many eco-physiological traits in response to elevated pCO_2 (Schaum et al. 2012). These results, along

with those presented in this study suggest that high variation in eco-physiological traits in response to pCO_2 may be an endemic feature of widely distributed oceanic microbes.

An important yet rarely tested follow up to the characterisation of eco-physiological variation is to explicitly ask if this variation is adaptive by making some estimates of fitness advantages of different genotypes in response to environmental change. Selection experiments (Barrick and Lenski 2013; Buckling et al. 2009) provide a solution as they can directly measure competitive fitness of extant genotypes under multiple environmental change scenarios, thus providing solid inference of adaptive responses from populations with standing variation.

Our understanding of the impacts of climate change on many marine species, and in some cases broad functional groups (Cripps et al. 2014; Hutchins et al. 2013), are often based on eco-physiological experiments performed with one or few strains of a species (Crawfurd et al. 2011; Dixson et al. 2010; Doo et al. 2011; Fu et al. 2008; Widdicombe and Needham 2007). Given that many species may exhibit high diversity in the responses of traits such as growth and size, it is likely that our inferences about these species may be a consequence of the selected genotypes used in the experiments. Meta-analyses conducted into biological effects of ocean acidification have often found variable effects on species (Hendriks et al. 2010; Kroeker et al. 2010), which is often cited as inter-specific variation but could also be explained by intra-specific variation or a combination of both. An important question relates to the relative contributions of inter and intra-specific variation to this apparent variability, as intra-specific variation points towards evolvability (Bolnick et al. 2011; Pauls et al. 2013), whereas inter-specific variability would suggest systematic species loss and the associated problems with ecosystem disturbance.

High variation in eco-physiological response is suggestive of adaptive potential (assuming a genetic basis): Higher standing variation leads to an increased likelihood of evolutionary rescue (Barrett and Schluter 2008; Bell and Collins 2008), as it both reduces the demand for novel mutations and provides a wider range of backgrounds in which those mutations can occur. Given that many marine microbes have wide distributions (Fenchel 2005; Watts et al. 2010), large population sizes, high dispersal potential (Finlay 2002), and high levels of functional and genetic diversity (Lowe et al. 2005; Lowe et al. 2012), it is reasonable to hypothesise microbes will have few problems in mounting an appropriate adaptive response to future environmental change (Joint et al. 2011). Nonetheless, identifying the potential source(s) of such adaptive diversity is important for predicting the composition of the future biosphere and to understand how non-microbial species will fare under climate change scenarios.

In contrast to my results, where I found no predictors of the response size or direction for either pCO_2 or temperature, the environmental background from which a genotype was derived can determine the response of organisms to climate change (Schaum et al. 2012; Thomas et al. 2012). There are many clearly documented examples of organisms from warmer regions expanding their ranges pole wards in both terrestrial and marine ecosystems (Chen et al. 2011; Perry et al. 2005; Sorte et al. 2010), though the picture is less clear for pCO_2. The temperature responses of *Oxyrrhis* strains in this study could not be predicted by mean annual temperature, sampling date temperature, mean spring temperature, or temperature variability. This could again be explained by the relatively high fine-scale temporal variability experienced by these organisms when compared to other marine species, but could also be a reflection of a low sample size (and thus statistical power) or an indication that the high level of genetic diversity at fine spatial scales (Lowe et al. 2010c) translates into high functional diversity; hence greater replication within sites is needed to further

understand response to changing climate. Schaum et al. (2012) found that for *O. tauri* the response to increased pCO_2 was explained by the levels of pCO_2 variability in the environment from which the strains were isolated, a relationship I was unable to detect in my data. Whilst there was substantial variation in levels of pCO_2 variability between sampling sites, there is a problem that these data are from coastal/oceanic sites rather than the intertidal waters that are the actual habitat of *Oxyrrhis*. Seawater contained in tide pools on rocky shores (where most of the strains were isolated) may follow different patterns in temperature and pCO_2, and is likely to be more temporally variable than the coastal seawater from which the environmental estimates were derived.

In addition to identifying the presence of high diversity, my results highlight some potential issues regarding the use of eco-physiological responses to infer species responses to climate change. One of the most important of these relates to evolutionary adaptation and the undeniable fact that typically microbial species evolve on ecological timescales (Selifonova et al. 2001). When assessing intra-specific variation and its response to environmental change we are attempting to infer fitness, however many eco-physiological process may not be directly related to fitness (Gillespie et al. 2008) and in cases where I would expect them to be (*e.g.* calcification in coccolithophores), it may not be clear what direction of response translates to increased fitness. One potential solution to this problem is to employ selection experiments (Collins 2010; Collins et al. 2014), to expand upon these by examining selection from standing variation in populations containing a mixture of strains, and to measure fitness directly under competitive scenarios (Chevin 2011), rather than inferring them from eco-physiological data.

Chapter 3

A rapid and cost-effective quantitative microsatellite genotyping protocol to estimate intraspecific competition in protist microcosm experiments

3.0 Introduction

There is a pressing need to understand the functional ecological consequences of intraspecific genetic variation. Intraspecific genetic diversity is predicted to have a fundamental impact upon a range of ecological and evolutionary processes (Bijlsma and Loeschcke 2005; Bolnick et al. 2011; Hughes et al. 2008), including population dynamics (Aguirre and Marshall 2012; Lankau 2009), ecosystem resilience to disturbance (Hughes and Stachowicz 2004), community function (Whitlock et al. 2007), adaptation from standing variation, and the impact of pathogens upon their hosts (Balmer et al. 2009). High genetic diversity can protect populations from extinction by facilitating evolutionary responses to environmental change (Markert et al. 2010), therefore intraspecific diversity could play a crucial role in conservation (Frankham 2005; Reed and Frankham 2003). Our understanding of the impact of intraspecific genetic diversity on such population processes is, however, limited by a lack of experimental data: existing experimental studies use either low or unquantified levels of intraspecific genetic diversity, and predictions (*i.e*. model parameterisation) tend to be based on the response of just one or relatively few genotypes (Yang et al. 2013).

Protists (eukaryotic microbes) are well-suited for experimental studies of ecological processes due to their fast growth rates, small size and the ease with which one may

manipulate and control experimental conditions (Collins and Bell 2004; Friman et al. 2008; Petchey et al. 1999). Moreover, protists are ecologically significant, underpinning numerous ecological processes, particularly in aquatic systems where protists drive primary production, nutrient cycling and recycling carbon via the microbial loop (Pernthaler 2005). High levels of intraspecific diversity is a feature of many natural protist populations (Evans et al. 2005; Harnstrom et al. 2011; Logares et al. 2009; Lowe et al. 2010c), however a lack of morphological features and small size makes distinguishing among protist strains within mixed cultures impossible without recourse to a molecular assay. Correspondingly, one aspect that has been largely overlooked in protist experimental systems is the precise role of intraspecific genetic variation. The individual contributions of separate strains to the experimental outcome is either not quantified or has to be estimated using a considerable re-sampling effort that imposes time and cost constraints: *e.g.* re-isolating many single cells/colonies from the experimental system to establish monocultures that are then individually genotyped to estimate strain frequencies (Roger et al. 2012).

In principle, the genetic composition of a sample can readily obtained by genotyping pool of DNA samples within a single PCR, whereby the frequency of each allele indicates the amount of genetic material that each sample contributed to the DNA pool (Gruber et al. 2002; Sham et al. 2002). For most purposes, DNA pooling is used to rapidly estimate population genetic parameters (e.g. differences in allele frequencies and diversity) at many loci and among several, large samples. Hence, contemporary protocols for genotyping DNA pools generally assess variation at single nucleotide polymorphisms (SNPs) (Doostzadeh et al. 2008; Gruber et al. 2002; Lavebratt and Sengul 2006; Sham et al. 2002) because of convenient genotyping methodology and as SNPs are the most abundant genetic marker in the genome (Schlotterer 2004). For model protists where whole genome data are available for multiple strains (e.g. species of *Saccharomyces*, *Chlamydomonas*), it may be

straightforward to identify many SNPs. Of course, next generation sequencing technology offers reasonable prospects for identifying SNPs in genomic non-model species (Nielsen et al. 2011; Peterson et al. 2012). However, where a study aims to follow the fate of many different genotypes during the course of an experiment, as opposed to quantifying population genomic parameters, it is typically difficult to identify short genomic regions (i.e. distinct and easily genotyped loci) that possess sufficient SNPs to be able to differentiate among more than just a few strains. For genomic non-model protist species, therefore, there is a need to develop a cost-effective and rapid molecular-genetic assay that can quantify the contributions of different strains to ecological outcomes from within experimental populations.

One such genotyping approach is to take advantage of the typically high polymorphism exhibited by microsatellite loci (Ellegren 2004; Schlotterer 2004); for example, in the eastern North Atlantic samples of the marine flagellate *Oxyrrhis marina* had between 6 and 36 different alleles at 9 microsatellite loci (Lowe et al. 2010c) but just three different haplotypes at ~900 bp of sequence data representing two distinct loci (Lowe et al. 2012). Indeed, microsatellite-based approaches to genotype DNA pools were developed to quantify diversity and differentiation among metazoan samples (Daniels et al. 1998; Hillel et al. 2003; Khatib et al. 1994; Skalski et al. 2006); under correct thermal cycling conditions, the amount of PCR product of every allele (as estimated from the electropherogram peak heights) reflects the amount of respective template DNAs. Hence, accurate genotyping of metazoan samples requires rigorous standardisation, in terms of sample quality and quantity, so that each sample makes a tangible contribution to the DNA pool and subsequent PCR. For example, Reininger et al. (2011) used the relative amplification of microsatellite alleles to estimate biomass of the endophytic fungus strains from environmental samples; this method used a reference strain, added to the sample prior to DNA extraction, to calculate each fungal species' biomass. By contrast, for protist samples the allele frequencies *per se* should indicate the relative numbers

of cells of each genotype (strain) within the mixed sample (Cruz et al. 2010; Takala et al. 2006).

The utilisation of eco-physiological fitness proxies is widespread in climate change experiments, where measurements such as growth or fecundity measured on single genotypes or populations in isolation are used to infer relative fitness in a way that conveys evolutionary meaning. Debate exists as to what are the best organismal traits for fitness inference, but few empirical studies have explicitly tested this. Selection coefficients (*s*) are a measurement of relative change in frequency of a genotype under competition and as such provide a direct measure of selection per unit time. They are estimated for a given strain i as:

$$s_i = \frac{d}{dt}\ln\left(\frac{p_i}{p_j}\right)$$

where p_i and p_j are the frequencies of strain i and j respectively. Selection coefficients can also be inferred from growth rates, as under conditions where genotypes do not interact (*i.e.* they all have the same growth rate as when grown in isolation) *s* for strain i is given by:

$$s_i = r_i - r_j$$

where r_i and r_j are the growth rates of strains i and j respectively (Chevin 2011). This gives us an opportunity to directly test how effectively eco-physiological rates predict selection, and will allow us to assess their suitability as fitness proxies in climate change experiments.

To study the dynamics of individual protist strains within mixed experimental cultures I (1) first describe the development of new microsatellite loci that can differentiate between multiple strains of *Oxyrrhis marina* and next (2) demonstrate how quantitative microsatellite genotyping can accurately determine the relative abundance of different strains from within mixed samples. I show that microsatellite loci can be used to accurately quantify the relative abundance of distinct alleles (*i.e.* different strains) in mixed samples. This easy, fast and

cost-effective technique opens up novel avenues for future research using protists to study intra-specific variation in experimental microcosms. I then implement this method to estimate instantaneous selection coefficients, compare these with selection coefficients derived from strain specific growth rates, and show that growth rates (*r*) do not predict fitness (*s*).

3.1 Methods & Materials

Cell culturing and DNA extraction

I examined strains of the marine flagellate *Oxyrrhis marina* Dujardin, 1895, a protist that is widely used in experimental studies (Lowe et al. 2010a; Montagnes et al. 2010; Yang et al. 2013). Seven strains of *O. marina* were isolated from the North East Atlantic Ocean: 47_BGN01, 47_BOD02, 47_TMO01, 44_PLY01, 33_ROS03, 351_FAR01 and 351_EST02. Stock cultures were maintained at 16°C in 32 PSU sterile filtered artificial sea water (ASW) enriched with f/2 (Sigma Aldrich, UK) and fed on *Dunaliella primolecta* at a cell density of ~3×10^5 cells ml^{-1}, and maintained at a light intensity of ~80 μmol photons m^{-2} and on a light:dark cycle of 14h:10h. Stock cultures were maintained by sub-culturing once per month. Approximately two weeks prior to DNA extraction, strains were sub-cultured in the dark and fed on heat-killed *E. coli* (Lowe et al. 2010b) to deplete *D. primolecta* (*i.e*. reduce the amount of non-target eukaryotic DNA).

To generate DNA for optimising microsatellite loci PCR conditions, *O. marina* cultures (400-500 ml) were grown to mid log-phase (5,000-10,000 cells ml^{-1}) and harvested by centrifugation. Total genomic DNA was extracted from cell pellets using a Qiagen Genomic-

tip 20/G (Qiagen) and the standard protocol for cell cultures. DNA concentrations were quantified using a Qubit dsDNA BR assay (Life Technologies) and samples were diluted to ~12.5 ng μl^{-1} and the DNA concentrations re-quantified in triplicate to ensure accuracy.

Microsatellite loci development

Microsatellite loci and corresponding primer sequences were obtained from an *O. marina* (reference strain 44_PLY01) RNAseq dataset (generated using Roche 454 sequencing – see (Lowe *et al.* 2011c)) using the QDD2 pipeline (Meglecz et al. 2010). I identified all unique loci that contained tri-nucleotide repeats, since this class of microsatellite loci typically suffers less from stutter banding than di-nucleotide repeats (Guichoux et al. 2011) and was more frequent than tetra- or penta-nucleotide repeats (data not shown). I then tested loci for PCR-amplification success and for polymorphisms using an M13 tailed primer method (Schuelke 2000) and by genotyping multiple strains of *O. marina*. Loci were amplified in 10 µl reaction volumes that contained Green GoTaq® reaction buffer (pH 8.5) (Promega), 200 µM each dNTP, 1.5 mM $MgCl_2$, 10 µg BSA, 0.25 units GoTaq® DNA polymerase (Promega), 0.3 pmol of forward primer (whose 5' end incorporated an M13 primer tail sequence), 0.3 pmol reverse primer and 0.3 pmol of an M13 primer that was 5'-labelled with either 6-FAM, NED, PET or VIC flourophores (Applied Biosystems) and ~25 ng target DNA. Thermal cycling conditions were: 94°C 3 min, 35×[94°C 30s, 58°C 30s, 72°C 45s] and 72ºC 4 min. PCR products were pooled with GENESCAN 500 LIZ (Applied Biosystems) and then separated by capillary electrophoresis on an AB3130xl (Applied Biosystems).

Quantitative microsatellite genotyping calibration

DNA from *O. marina* strains was mixed into a series of DNA pools to provide a combination of eight pairs of strains, each with twelve ratios of DNA (that varied between 50:1 and 1:50).

The following strain combinations were tested: (1) 351_FAR01 & 47_BOD02; (2) 33_ROS03 & 351_EST02; (3) 33_ROS03 & 47_BGN01; (4) 351_EST02 & 47_BOD02; (5) 44_PLY01 & 47_BGN01; (6) 47_BGN01 & 47_TMO01; (7) 47_BGN01 & 33_ROS03; and (8) 47_TMO01 & 44_PLY01. Expected DNA ratios were calculated using the mean DNA concentrations and the proportions in which the DNAs were pooled.

All test pools of DNA were PCR-amplified (*n*=8 replicates) using one of four microsatellite loci. For these quantitative PCRs, the forward primers were labelled with a 5' fluorescent dye (Table 3.1.) and the PCR used the standard two (forward and reverse) primers. Loci were amplified as 10 μl PCRs as described above (*i.e.* with GoTaq® DNA polymerase [Promega]), but with 0.3 pmol of forward primer (5' labelled with either 6-FAM, NED, PET or VIC, Applied Biosystems) and 0.3 pmol reverse primer. Thermal cycling conditions were: 95°C for 1 min, N_c×[95°C 30 s, 58°C 45 s, 72°C 45 s], 72°C 10 min, where N_c represents the number of cycles (Table 2). PCR products were pooled with GENESCAN 500 LIZ size standard (Applied Biosystems) and separated by electrophoresis on an AB3130xl (Applied Biosystems).

Further calibrations were performed using DNA extracted with Chelex-100™ (Walsh et al. 1991). Here, the target DNA was a series of mixed cultures to simulate a protist microcosm

Table 3.1. Microsatellite primers and loci information developed and used for quantitative genotyping in this thesis, Product size refers to the reference strain (44_PLY01).

Locus ID	5' Fluorescent dye	Motif	Product size	N_c	5' Primer sequence	3' Primer sequence
MS_23	6-FAM	AGC	258	33	CTTGCAACACCAAGTCAAAGTG	CTTCCAAATATCTGTTGCGGTC
MS_30	VIC	GAT	163	32	CTGTGGATGACAGCGATGAG	GTACCAGAAGTGAACCAATGGC
MS_37	NED	CGC	136	29	GATGACTTCCGTGCGTATCTTC	CTGAACCTTGACTACCAAACGG
MS_41	PET	TGC	119	32	ATCGGTCTGGAACAATTGGAAG	TCGTCGCGATCGTAGGAGAG

experiment: *O. marina* cultures were diluted to 1,500 cells ml^{-1} the strain pairs (1) 33_ROS03 & 351_EST02, (2) 351_EST02 & 47_BOD02, (3) 44_PLY01 & 47_BGN01, and (4) 47_BGN01 & 47_TMO01 then mixed at four ratios of 1:24, 1:4, 4:1 and 24:1. Samples were centrifuged and genomic DNA extracted from the cell pellet by adding 50 μL Chelex-100™ (Bio-Rad Laboratories) solution (5% w/v) and then incubating the samples at 95°C for 1 hour. PCR amplification of four microsatellite loci (MS_23, MS_30, MS_37 and MS_41) was performed for each sample (and in replicate n=8) as described above, but with 2 μL of the Chelex-100™-extracted DNA as a template DNA. Genotyping was performed as described above. Expected ratios of the pair of strains were calculated from the mix ratio and a triplicate cell density count of each population, performed using a Sedgewick Rafter Chamber.

Multi-strain genotyping calibration

I also tested the ability of microsatellite genotyping to quantify the relative abundance of four strains of *O. marina* from a mixed population. Stock cultures of 47_BGN01, 47_TMO01, 33_ROS03, & 44_PLY01 were diluted to approximately 1,000 cells ml^{-1} and mixed in twelve combinations of four ratios (25:13:8:3), where at any given ratio for a specific strain there were three different combinations of the other strains' specific ratio. Cells were harvested by centrifugation and DNA was extracted using Chelex-100™. Microsatellite alleles at locus MS_41 were amplified by PCR and genotyped using the same methods as described for pairs of strains.

Growth rate experiments

Growth rates were estimated for each individual strain in triplicate, in 45 mL SASW medium and heat killed *E. coli* in 50 mL centrifuge tubes. Strains were independently inoculated at a cell density of ~500 cells mL^{-1} and sampled after mixing at 0 & 48 hours by fixation of 5 mL

of culture in 2% Lugol's iodine. Cell densities were estimated manually using a Sedgewick Rafter Chamber on a Zeiss Axiovert A1 inverted microscope (Carl Zeiss, Germany). Growth rates (*r*) were calculated as the slope of natural log density with time.

Selection coefficient experiments

Selection coefficients (*s*) were estimated directly under competition for 12 pairs of strains by inoculating two strains at a total cell density of 500 cells mL^{-1} and at an equal frequency (*i.e.* 250 cells mL^{-1} for each strain) in triplicate. Initial culture volumes were made up to 50 mL with SASW and heat killed *E. coli*. After mixing 10 mL samples were taken from each microcosm at 0 and 48 hours in 15 mL centrifuge tubes, centrifuged immediately at 4500 rpm and 4 °C for 30 minutes. After removing supernatant pellets were frozen at -20 °C for up to 3 days before DNA extraction.

Data analysis

Allele sizes and peak heights were determined using Genemapper v.3.0 (Applied Biosystems). These data were then transformed to a peak height ratio (PHR) - the ratio between the peak heights of the alleles generated by the two strains - which was natural log-transformed prior to analysis in all cases. Calibration curves were created using linear least-squares regression (Levenberg-Marquardt Algorithm). Multi-strain calibration was performed using multiple-regression, with one model for each strain. Additional terms were included for ratios of non-target strains, followed by step-wise removal of non-significant terms until a minimal model was found. Selection coefficients (*s*) for a target strain versus a non-target strain were estimated from the slope of log strain ratio with time. Growth rates were calculated as the slope of natural log density over time. For each strain pair in which a direct estimate of *s* was obtained from selection experiments, a growth based selection coefficient was also estimated by subtracting the non-target strain's growth rate from the

target strain's growth rate (Chevin 2011). Correlation between the two estimates of *s* were analysed by linear least squares regression. All statistical analyses were performed using R v.2.15.3 (R Core Development Team, 2013).

3.2 Results

Microsatellite loci identification

I identified some 130 microsatellite loci from Lowe et al. (2011)'s transcriptome data for *O. marina*. Out of the 38 trinucleotide microsatellite loci (for which 47 primer pairs were designed), ten loci amplified clean, single peaks for each allele and presented no PCR-failures, potential null alleles or substantial stutter banding (Guichoux et al. 2011; Selkoe and Toonen 2006). Out of these ten loci, four were homozygous and polymorphic in at least some of the strains tested (*i.e.* loci exhibited just one allele within strains, but with alleles differing among strains), and thus suitable to quantify strain abundance in mixed cultures of *O. marina*. The final panel of loci used for quantitative genotyping (referred to as MS_23, MS_31, MS_37 and MS_41) yielded 3, 4, 2 and 7 alleles respectively in the eight strains of *O. marina* that I tested.

Pairwise quantitative genotyping

Quantitative microsatellite genotyping effectively recovered the known genotype frequencies from the test pools of DNA (Fig.3.1., Table 3.2.). Calibration curves fit the data very well in most cases (R^2>0.97), with the regression slopes usually close to 1 (range 0.84-1.18) and the intercepts mostly close to 0, albeit with some statistically significant differences from expected values (Table 3.2.). Detection and quantification of alleles was possible at nDNA frequencies as low as 0.02 (i.e. 1:50 ratio of cell abundance). Based on analyses of residuals

I found no systematic bias in the error distributions of the fitted linear models to the data. Calibrations using DNA extracted with Chelex-100 were successful also (Fig.3.1.), producing values of R^2>0.99 for all four trials. In addition, data for peak height ratio matched those obtained from pooled DNA calibrations relatively well; hence, this microsatellite-genotyping technique works with potentially low quality DNA samples that are obtained when using a cheap and fast extraction method.

Table 3.2. Quantitative genotyping calibration regression statistics.

Strain A	Strain B	Marker	A size (bp)	B size (bp)	R^2	Slope	Intercept
351_FAR01	47_BOD02	MS_23	338	344	0.984	0.990	-0.384
33_ROS03	351_EST02	MS_23	341	344	0.997	1.180	0.174
33_ROS03	47_BGN01	MS_23	341	344	0.973	0.933	0.487
351_EST02	47_BOD02	MS_30	162	168	0.988	0.957	0.686
44_PLY01	47_BGN01	MS_37	136	142	0.960	0.841	1.411
47_BGN01	47_TMO01	MS_41	92	101	0.994	1.031	0.083
47_BGN01	33_ROS03	MS_41	92	113	0.993	0.927	1.435
47_TMO01	44_PLY01	MS_41	101	119	0.970	0.925	0.229

Multi-strain calibration

Multi-strain calibration was successful, but required multiple-regression estimated calibration coefficients that took account of the ratios of non-target alleles in the data (Table 3.3.). Regression models fitted the data well, with R^2 values of 0.96, 0.93, 0.92, & 0.96 for 47_BGN01, 47_TMO01, 33_ROS03, & 44_PLY01 respectively.

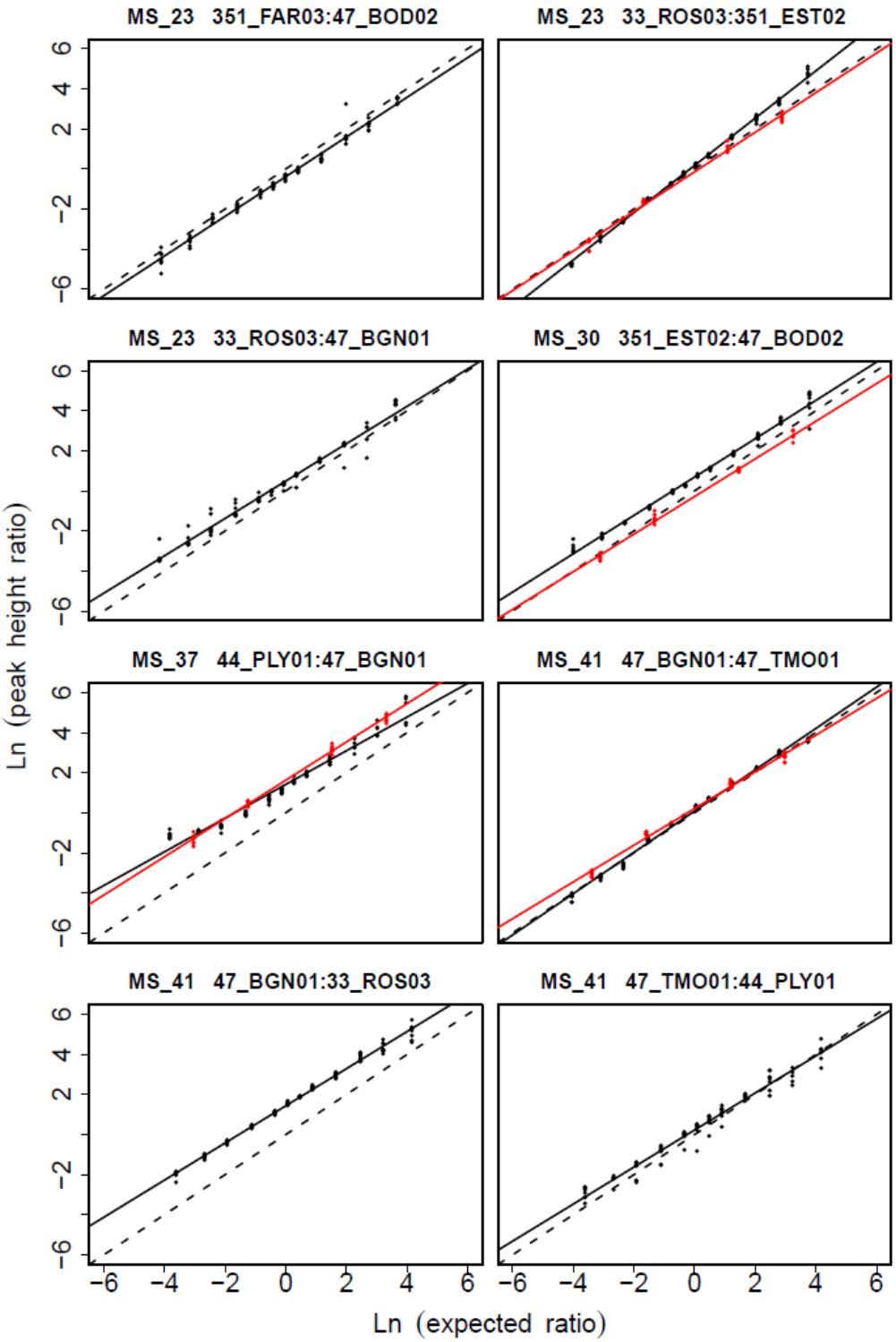

Fig.3.1. Quantitative genotyping calibration curves for eight pairs of strains. Black data are derived from samples of extracted nDNA mixed in known ratios; red data are for known ratios of mixed cells. Solid lines are regression fit to data, dashed lines are y=x.

Table 3.3. Multiple-regression statistics for quantitative genotyping of four strains of *Oxyrrhis*

Response	Coefficients	β	s.e.	p	Multiple R^2
ln(47_BGN01)	Intercept	-1.05	0.11	1.70E-12	0.9582
	ln(expected)	0.42	0.03	< 2.00E-16	
	ln(47_TMO01)	-0.48	0.05	4.31E-13	
	ln(44_PLY01)	-0.26	0.04	1.00E-07	
ln(47_TMO01)	Intercept	-0.38	0.24	1.10E-01	0.9354
	ln(expected)	0.49	0.06	3.58E-10	
	ln(47_BGN01)	-0.27	0.08	2.14E-03	
	ln(44_PLY01)	-0.26	0.06	8.65E-05	
ln(33_ROS03)	Intercept	-1.42	0.06	< 2.00E-16	0.9240
	ln(expected)	0.85	0.04	< 2.00E-16	
ln(44_PLY01)	Intercept	-1.65	0.11	< 2.00E-16	0.9657
	ln(expected)	0.51	0.04	< 2.00E-16	
	ln(47_BGN01)	-0.54	0.06	7.02E-12	
	ln(47_TMO01)	-0.72	0.05	< 2.00E-16	

Growth and directly estimated fitness

There was no significant relationship (Regression, F_{10}=0.50, R^2= 0.048, P=0.5) between directly estimated *s* and growth rate estimated *s* (Fig.3.2.). A weak positive correlation was present in the data (Pearson's correlation coefficient = 0.22), however in five out of twelve cases growth rates predicted the incorrect direction of selection observed under competition and in five of the other seven cases the magnitude of selection was significantly different from that predicted by growth rates. Only two out of twelve directly estimated *s* were predicted by growth rate estimated *s*.

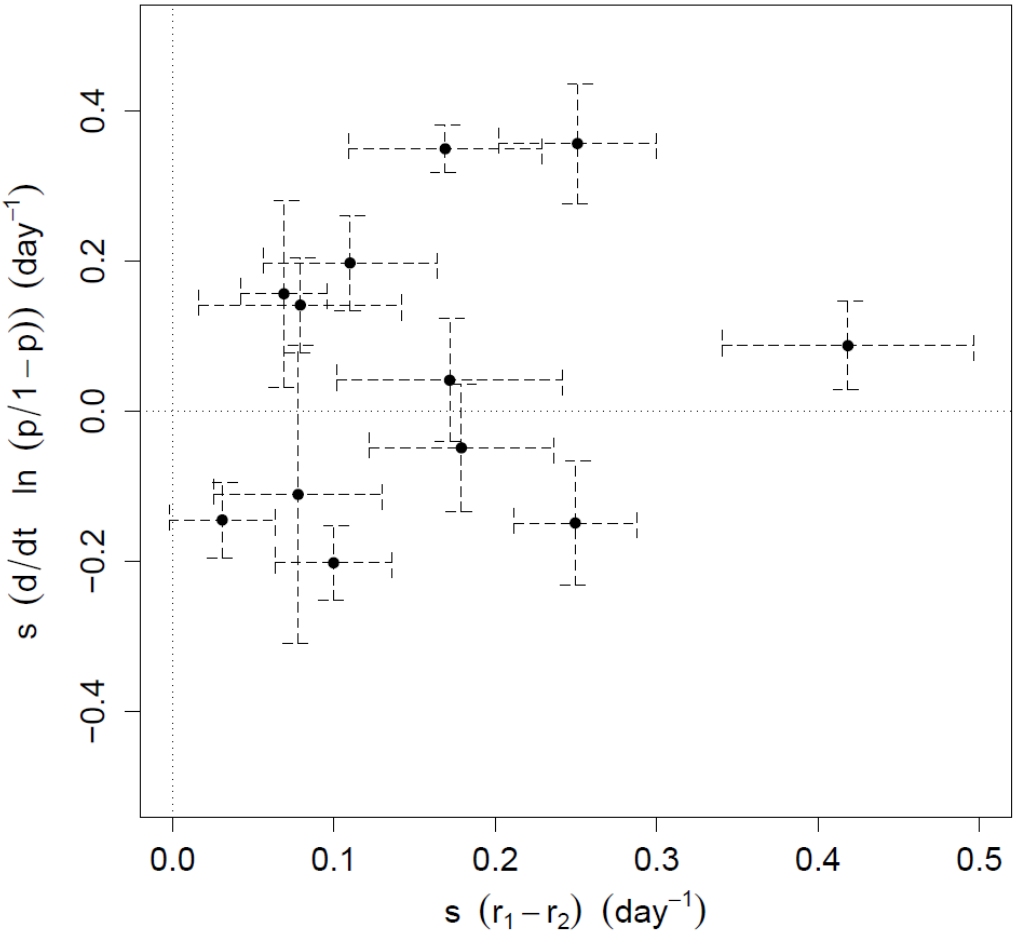

Fig.3.2. Association between selection coefficients (*s*) for pairs of *Oxyrrhis* as estimated directly under competition and by subtracting growth rates. Error bars indicate ± 1 SE.

3.3 Discussion

Species naturally exhibit more genetic diversity than can be examined during laboratory and field experiments. Yet understanding the consequences of how the variation that exists within and between populations may interact is of fundamental importance to understanding numerous ecological and evolutionary processes (Bijlsma and Loeschcke 2005; Hughes et al. 2008). Here I demonstrate how quantitative microsatellite genotyping can be used to determine the relative abundance of multiple protist strains that are interacting within a mixed

population, and without a need for high-quality DNA extraction or quantification. Once microsatellite loci are available, therefore, this method provides a fast, cheap, and easy-to-implement way of studying intra-specific dynamics of many, genetically different protist strains within experimental microcosms.

Microsatellite loci are good genetic markers to differentiate among strains of protists for a number of reasons. First, they are present in the genomes of nearly all known eukaryotes (Aishwarya et al. 2007; Schlotterer 2004). Second, microsatellites are often highly polymorphic (Ellegren 2004; Guichoux et al. 2011; Schlotterer 2004), which makes them capable markers of intra-specific diversity; for example, within British coastal waters, more than 180 out of 200 isolated strains of *O. marina* had unique genotypes at 9 microsatellite loci (Lowe et al. 2010c), whereas the samples from the same region were monomorphic for sequences of cytochrome c oxidase I and α-tubulin (Lowe et al. 2012). Other studies typically find between 10 and 20 alleles at most microsatellite loci during field-surveys of protist intraspecific diversity (Evans et al. 2005; Harnstrom et al. 2011). Nonetheless, some pertinent issues when developing microsatellites should be noted. I targeted tri-nucleotide microsatellite loci, as they were predicted to suffer less from stutter banding than di-nuclotide repeats (Guichoux et al. 2011). However, stutter banding is neither limited to, nor an inherent problem of, di-nucleotide microsatellite loci; indeed the fungal biomass estimates were derived from allele frequencies at dinucleotide microsatellite loci (Reininger et al. 2011). Thus, other studies may use di-nucleotide loci as a means of finding greater numbers of presumably more polymorphic loci. For practical purposes, I found that using peak height ratio gave slightly better estimates than peak area ratio (based on regression R^2 estimates; data not shown), but this difference was negligible. For species without characterised microsatellite loci, next generation sequencing data provide a rapid means of isolating many microsatellite loci (Fernandez-Silva et al. 2013; Meglecz et al. 2004).

I was able to accurately estimate the relative frequencies of multiple strains within mixed pools using a simple Chelex-100 protocol to extract DNA from a mixed cell culture, almost as well as using the more time-consuming and expensive DNA extraction kit. Extracting DNA directly from a mixed sample, rather than having to re-isolate and individually genotype cells (Roger et al. 2012) saves time and money, thereby opens up the possibility of tracking the dynamics of specific protist strains in experimental microcosms that contain high standing diversity. By extension, it might be informative to use this genotyping method to assess allelic diversity and differences between samples of natural populations. At present, protistologists spend considerable effort isolating single cells from environmental samples for genotyping (Evans et al. 2005; Harnstrom et al. 2011; Lowe et al. 2010c). However, preliminary genotype data using the DNA extracted from environmental samples, possibly with some culturing (depending on the cell density of target the species in the environmental sample) but without isolating and genotyping single cells *per se*, may provide baseline information about the distribution of genetic diversity over a wider area. This information would then allow a more-informed sampling strategy that targeted 'interesting populations' for detailed genotyping of single cells.

An assumption of this methodology is that number of genome copies of the genetic marker(s) correlates with numbers of individual cells, and thus there may be problems in calibrating loci that have been duplicated (Meglecz et al. 2007) or in species where the DNA content per cell varies among individuals (Parfrey et al. 2008). Nonetheless, this study highlights an obvious time and cost benefit associated with using a protist model where the focus is on single cells. For quantitative genotyping studies of metazoans, every individual's DNA needs to be extracted alone due to varying cell content per individual and then the DNA concentrations per sample have to be standardised prior to constructing DNA pools. While there is no need to identify an appropriate reference strain for this method (Reininger et al.

2011), it is advisable to initially culture and genotype individual strains so that standard pools of DNA (or mixed cells) can be used to calibrate and validate the assay. An additional advantage is that suitably polymorphic microsatellite loci are available for many protists. If unavailable, they can be easily found by mining expressed sequence tag (EST) or other genomic datasets, of which many exist for model and non-model protist species.

Microsatellite-based genotyping of DNA pools (Daniels et al. 1998; Hillel et al. 2003; Khatib et al. 1994; Skalski et al. 2006) has largely been replaced by other methods to estimate genotype frequencies from mixed populations of cells, including qPCR melt curve analysis (Cruz et al. 2010), SNP based pyrosequencing (Wasson et al. 2002), and FREQ-Seq (Chubiz et al. 2012). Whilst these methods typically present high levels of accuracy and the possibility to detect alleles at a lower frequency than the microsatellite method, they all suffer the same problems when working in non-model organisms that have large and complex genomes (*i.e.* most protists): they all require identification of SNPs between the genotypes that are to be quantified, and finding suitable loci with limited genomic resources is both time consuming and expensive. My method fills this gap by employing microsatellite loci that are both easily identifiable and highly polymorphic across eukaryotic taxa. Also, my method works well with relatively 'dirty' DNA samples (i.e. extracted using Chelex-100) and it is particularly fast and cheap.

One important application of this method is to better understand natural selection - the dynamic process by which a particular genotype (or allele) increases in relative frequency in a population due to higher fitness (Chevin 2011). While the outcome of selection, and hence evolution, is determined by competition between genotypes of the same species, the contribution that apparently simple parameters, such as growth rate (r) and carrying capacity (K), make to fitness and selection, surprisingly remains widely untested by empirical research

(Chevin 2011). The ability to infer fitness from experimental evolution / selection experiments has wider application, especially in the fields of global change biology (Collins et al. 2014), drug resistance evolution (Habets and Brockhurst 2012), and agricultural pest management (Lopes et al. 2008). Traditionally these fields have employed prokaryote, plant, or animal models, but in all three areas wider taxonomic coverage and more functionally relevant studies should encompass a greater emphasis on protists. This is especially true when considering the marine planktonic ecosystems, where single celled eukaryotes account for the majority of primary productivity and play a considerable role in the major biogeochemical cycles. Protists exhibit substantial functional and genetic diversity (Kremp et al. 2012), but a major challenge is to understand the relevance of this diversity in mixed populations. Using microsatellites to follow the success and demise of pre-defined genotypes (strains) under competition will allow us to make inferences about how populations with high standing genetic variation may adapt to future environmental change, and thus shape the future biosphere.

Many evolutionary studies infer relative fitness of genotypes within a species using measurements of eco-physiological rate processes such as growth or with life history traits such as fecundity on the individual genotypes cultured in isolation (Brommer 2000). Here I show that growth based fitness proxies are inconsistent with those observed when disparate genotypes are co-cultured, which suggests that interactions between genotypes under competition are prevalent (Chevin 2011). By using two methods (direct and indirect) to estimate the same parameter (*s*) I expect that if each genotype grows at the same rate under competition as in isolation I should derive the same value of *s*. This was rarely the case in my data (two out of ten) highlighting the complex relationship between growth and relative fitness. Interactions between genotypes could arise from two sources: inhibition of one strain by the other under competition, or stimulation of growth in one strain in the presence of a

competitor. There is limited experimental data that investigates the relationship between growth and fitness, but it generally supports my observation that one does not predict the other (Crone 2001; Zeyl et al. 2001), and in plant pathogens often there is a negative association (Newton et al. 1997; Welz and Leonard 1993; Wille et al. 2002). It has been long recognised that growth may not relate well to fitness, however most studies consider fitness as measured over longer timescales and in relatively complex organisms and ecological scenarios (Coulson et al. 2006), whereas here I show that even over a relatively short incubation period (2 days) and in an asexual population of single cells in pure culture, this still holds. The implication is that for making assessments of adaptation to climate change from standing genetic variation, eco-physiological estimates of fitness may be misleading or entirely inappropriate unless evidence exists that traits measured are directly linked to evolutionary fitness.

The results presented here have obvious implications for eco-physiological inference of climate change responses and for the use of selection coefficients as alternative proxies. Unlike other environmental impacts on marine ecosystems, such as oil spills, climate change is a relatively slow change in conditions which could allow for evolutionary responses by natural populations. Put simply, eco-physiological approaches cannot account for adaptation: they test organisms adapted to the present conditions in simulated future conditions. To account for evolution when examining a species' response, I should be asking how the present genotypic variation may be selected to adapt to the future. My results here suggest that eco-physiological data may provide misleading inferences, as growth rates are clearly demonstrated to be poor predictors of fitness. The selection coefficient (or competitive fitness) provides an alternative as it directly estimates fitness of competing genotypes by quantifying the rate of change in frequency of a genotype relative to another.

Chapter 4

Negative frequency dependent selection is intensified at higher densities in *Oxyrrhis marina*

4.0 Introduction

It has long been recognised that ecology effects evolution (Hutchinson 1965), but more recently a greater focus has been placed on how evolution affects ecology (Schoener 2011). The ability for each to reciprocally affect the other is gathering empirical support (Agrawal et al. 2013; Turcotte et al. 2011; Yoshida et al. 2003) and would indicate that evolution cannot be ignored when asking ecological questions (Reznick 2013) a situation that has important consequences for a range of questions in ecology. Frequency dependence and density dependence of fitness, and also selection, are potential examples of reciprocity between ecological and evolutionary processes, yet the two have rarely been examined in combination in either theoretical (Eadie and Fryxell 1992; Newton et al. 1998) or empirical studies (Levitan and Ferrell 2006; Mappes et al. 2008).

Whilst ecological work tends to emphasise density dependent selection, evolutionary studies usually are more focussed on frequency dependent selection (Kokko and Lopez-Sepulcre 2007). Such bifurcation in emphasis likely reflects the historical view that ecological and evolutionary dynamics occur on very different timescales (Slobodkin 1961): hence, whilst population densities are often observed to vary with high periodicity over somewhat short timescales (Rikalainen et al. 2012), it is often assumed that genotypic frequencies or trait distributions change much more slowly. As more examples of rapid contemporary evolution

are observed (Koskinen et al. 2002; Reznick et al. 2004), it is clear that the timescales of ecological and evolutionary dynamics often overlap. The corollary is that we require a more detailed and integrated understanding of how density and frequency interact to affect selection (or fitness).

Density dependent selection presents a fundamental link between ecology and evolution (Travis et al. 2013) as it represents an interaction between two of the most basic units of either dynamical process: number of individuals and genotype frequencies respectively. The idea that fitness of genotypes within a population may vary with population density (Ayala 1965; MacArthur 1962) suggests a genetic trade-off between fitness under different density regimes that could be involved in evolutionary divergence of lineages and speciation (Schluter 2000) as well as maintaining diversity in life history traits (Mueller 1997). These two consequences are closely linked to the concepts of *r*- and *K*- selection (Joshi et al. 2001), where opposing traits are selected at different population densities.

Frequency dependent selection may be common in natural systems (Levin 1988) and its fundamental importance is the maintenance of genetic diversity (Judson 1995). Negative frequency dependent selection is a process that favours rare genotypes (or strategies) and is implicated as the mechanism behind maintaining polymorphisms (Svensson et al. 2005) as well as in driving ecological diversification (Friesen et al. 2004). Frequency dependent selection can also be positive (Endler and Mappes 2004) whereby common genotypes are favoured by selection, with the potential outcome to erode genetic diversity. Common examples of positive frequency dependent selection tend to have a behavioural basis, for example in runaway sexual selection (Fisher 1930), or where a common behaviour is necessary for example in aggregative defence (Marples and Mappes 2010). Negative frequency dependence appears to be relatively more prevalent and is especially important in

antagonistic co-evolution (Dieckmann 2002), as counter-adaptations are most strongly selected for against common genotypes in opponent populations (Ebert 2008), thus favouring fitness of the rarer opponent genotypes.

A largely untested question, however, is 'how does genotype frequency interact with population density? Resolving this question is important because, as discussed above, both processes are relevant in natural populations and can impact selection. There is a growing appreciation of the need to integrate eco-evolutionary interactions (Kokko and Lopez-Sepulcre 2007; Reznick 2013; Schoener 2011) and understanding the role and interaction between frequency- and density- dependent selection is likely to be fundamental within this theoretical framework. For example, a model by Newton et al. (1998) showed that by adjusting simple life history parameters, selection could be either density- or density- and frequency- dependent, with their simulations implying that frequency dependence mattered more at higher population densities. The few empirical studies that have explicitly tested for an interaction between frequency and density, indicate that an interaction may be present (Levitan and Ferrell 2006; Mappes et al. 2008) in agreement with simulations (Newton et al. 1998) that frequency dependence may be more important at high densities. One limitation with these empirical studies is that they examined one-off interactions between a single pair of phenotypes (or genotypes). More comprehensive and wide ranging data are required to assess the generality of the evidence for an interaction between frequency- and density-dependent selection.

In this chapter I experimentally test for frequency- and density- dependent selection between six pairs of strains of *Oxyrrhis marina*, grown in mixed cultures, by measuring selection coefficients (Chevin 2011) from short incubations under a range of initial frequencies and population densities in a full factorial design. While density and frequency interact to affect

selection rates so that negative frequency dependent selection is stronger at higher population densities and almost absent at low densities. Moreover, there is high variation between pairs of strains in the responses of selection to frequency and density.

4.1 Methods & Materials

Experimental organisms and stock culturing

I explored instantaneous fitness measurements using eight strains of the model flagellate *Oxyrrhis marina* Dujardin 1895, isolated from European coastal sites on the North Atlantic Ocean. The strains are referred to as 351_EST01 (Estoril, Portugal), 351_FAR03 (Faro, Portugal), 33_ROS03 (Roscoff, France), 44_PLY01 (Plymouth, UK), 44_WIR02 (Wirral, UK), 47_BGN01 (Bergen, Norway), 47_BOD01 (Bodo, Norway), & 47_TMO01 (Tromso, Norway).

Strains of *O. marina* were isolated from seawater samples taken from tide pools on rocky shores and maintained at 16°C at a light intensity of ~80 μmol photons m^{-2} on a light:dark cycle of 14:10. Media was 32 PSU sterile filtered artificial seawater (SASW) enriched with f/2 (Sigma Aldrich, UK) and inoculated with *Dunaliella primolecta* at a cell density of ~3×10^5 cells ml^{-1} as a prey. Stock cultures were sub-cultured once per month. Pre-experimental cultures were created at least one month prior to experiments, without addition of f/2, and by replacing *D. primolecta* with heat killed *Escherichia coli* (Lowe et al. 2010b) at a density of 1.25-2.5 × 10^6 CFU ml^{-1}. Depending on *Oxyrrhis* density, fresh food was added to cultures every 2-5 days.

Frequency-density effects on selection experiments

Selection experiments to test for frequency and density dependence were performed by growing six pairs of strains within microcosms for a short period of time (2 days) in order to estimate instantaneous selection coefficients (Chevin 2011). The experimental microcosms were initiated at three initial frequency treatments (0.1, 0.5, 0.9) of the target strain and three total population density treatments (500, 2000, 5000 cells ml^{-1}) in a full factorial design with replication for each combination (n=3). Microcosms were established in 50 mL centrifuge tubes in SASW media, fed heat killed *E. coli* at a density of ~ 1.25-2.5 × 10^6 CFU ml^{-1}. After mixing by inversion 10 mL samples were taken from each microcosm at 0 and 48 hours in 15 mL centrifuge tubes, centrifuged immediately at 4500 rpm and 4 °C for 30 minutes. After removing supernatant pellets were frozen at -20 °C for up to 3 days before DNA extraction.

DNA Extraction and quantitative genotyping

To measure genotype frequencies, I used a quantitative microsatellite genotyping method, as developed in Chapter 2. Frozen DNA pellets were defrosted and washed twice in PBS. Genomic DNA was extracted from the cell pellet by adding 50 µL Chelex-100™ (Bio-Rad Laboratories) solution (5% w/v) and then incubating the samples at 95°C for 1 hour. Microsatellite loci were amplified by polymerase chain reaction in replicate (n=4) using one of four microsatellite primer pairs for each combination of strains (see Chapter 3). PCR reactions contained 2 µL of the Chelex-extracts as a template DNA in 10 µl reaction volumes that contained Green GoTaq® reaction buffer (pH 8.5) (Promega), 10 µg BSA, 0.3 pmol of forward primer (5' labelled with either 6-FAM, NED, PET or VIC, Applied Biosystems), and 0.3 pmol reverse primer. Thermal cycling conditions were: 95°C for 1 min, N_c×[95°C 30 s, 58°C 45 s, 72°C 45 s], 72°C 10 min, where N_c represents the number of cycles (see Chapter 2).

PCR products were pooled with GENESCAN 500 LIZ size standard (Applied Biosystems) and separated by electrophoresis on an AB3130xl (Applied Biosystems).

Data analysis

Microsatellite alleles were scored using Genemapper v.3.0 (Applied Biosystems) and peak heights were measured for each allele belonging to the strains used in each selection experiment. The peak height ratio was log-transformed and for each sample a strain ratio was calculated using a strain pair specific calibration curve (sees Chapter 3). Selection coefficients (*s*) for a target strain versus a non-target strain were estimated from the slope of natural log strain ratio with time (Chevin 2011).

A global model of selection coefficients was analysed using a mixed effects model, with random slopes and *s* as the dependent variable, density and frequency as fixed effects, and strain pair as a random effect using the R package 'lme4' (Bates et al. 2014). Due to the non-independent and reciprocal nature of selection coefficients (where for a given pair of strains the value of *s* for the focal strain is equal to the negative value of *s* for its competitor) the strain with the positive mean *s* across treatments was designated the test strain. To investigate interactions between density and frequency, further analyses were performed independently on frequency dependence within density treatments, by ANOVA, using a simple main effects test with an adjusted α value of 0.017. Frequency and density dependence on selection coefficients was also tested for each pair of strains independently by two-way ANOVA, with *s* as the dependent variable and frequency and density as factors. Two strains from disparate sampling environments (351_EST01 & 47_BGN01) were further analysed to test within a single target strain the effects of different non-target strains. All statistical analyses were conducted in R v.3.1.0 (R Core Development Team, 2014) and all data are presented as means ± 1 SE.

4.2 Results

Frequency and density dependent selection

Across all experiments there were significant interactions between frequency and density, frequency and strain pair, and density and strain pair on selection coefficients (Mixed effects model, χ^2=26.7, d.f.=4, p<0.001). The main effect of the interaction between frequency and density is explained by weak or a lack of significant frequency dependence at low (Simple main effects ANOVA, $F_{2,51}$=3.30, P=0.048) and medium (Simple main effects ANOVA, $F_{2,51}$=0.19, P=0.83) population densities and strong, significant negative frequency dependent selection at high population densities (Simple main effects ANOVA, $F_{2,51}$=10.16, P<0.001, Fig.4.1).

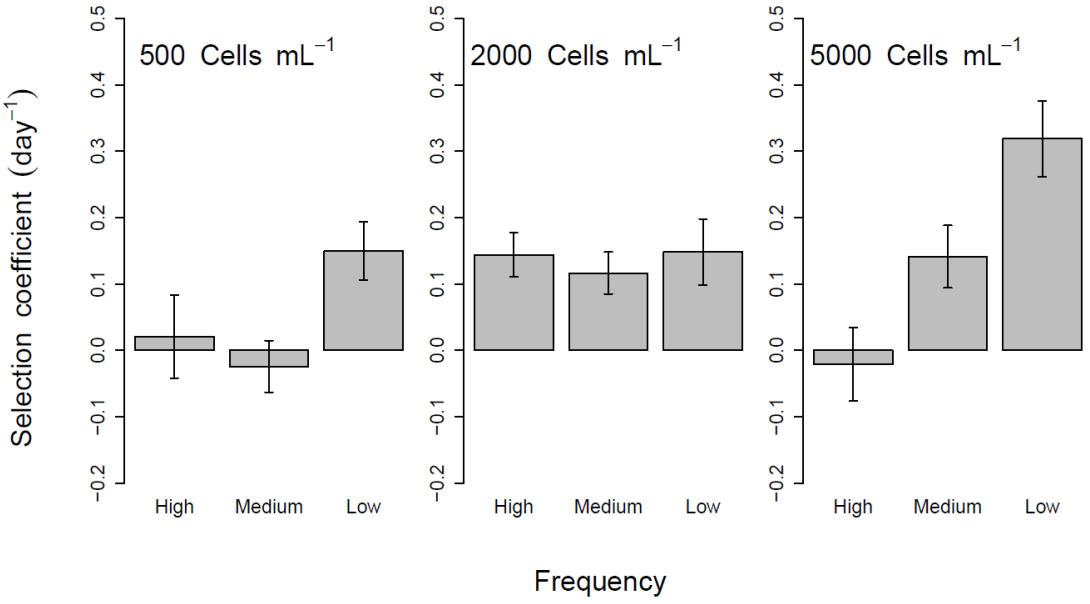

Fig.4.1. Mean selection coefficient for seven strains of *Oxyrrhis* relative to a competitor at low (500), medium (2000), and high (5000 cells mL^{-1}) population densities (panels) and low (0.1), medium (0.5), and high (0.9) initial frequencies (bars).

Due to the arbitrary nature used to select a strain as 'focal' (i.e. based on it having a mean positive selection coefficient) I tested for competitor specific effects by assigning two focal strains. For these focal strains I observed similar responses of selection to frequency and density: For 351_EST01 there was an interaction between frequency and density on selection (Two-way ANOVA, $F_{4,70}$=3.188, P<0.05) that followed the pattern described above, but with no interaction with competitor strain, suggesting that 351_EST01 responded to changes in frequency and density regardless of its competitor. For 47_BGN01, there was a three way interaction between density, frequency, and the competitor (Three-way ANOVA, $F_{8,54}$=2.248, P<0.05) that suggests a competitor specific response to frequency and density for this strain.

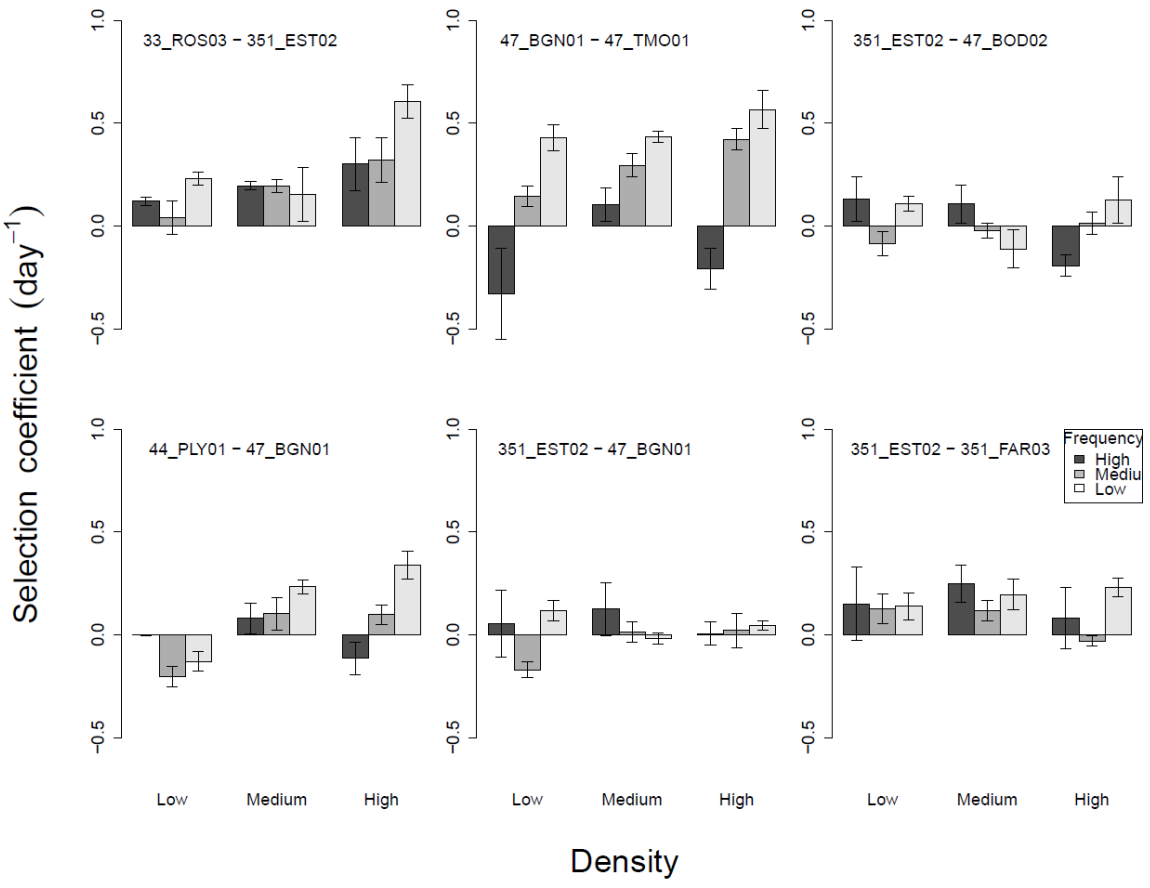

Fig.4.2. Strain pair (topleft of each panel) specific frequency- and density-dependence of selection coefficients (s). The first strain named is the target strain; the second is its competitor.

Due to the high variability of responses between strain pairs I also analysed the effects of frequency and density for each individual strain combination (Fig.4.2.). The experimental microcosms revealed complexity with all possible combinations of frequency dependence (47_BGN01 & 47_TMO01), density dependence (33_ROS03 & 351_EST02), and interactions between density and frequency dependence (44_PLY01 & 47_BGN01; 351_EST02 & 47_BOD02), and also two pairs of strains (351_EST02 & 47_BGN01; 351_EST01 & 351_FAR03) showing no significant effect of frequency or density on selection (Table 4.1.). The implication is clear: the type of response exhibited to variation in frequency and density depends upon the combination of genotypes.

4.3 Discussion

Natural selection is the fundamental process by which populations adapt to novel environments and forms the basis of understanding evolutionary dynamics. However, selection may be context dependent, and vary throughout time in response to a host of environmental and biological drivers. Very few experimental studies have attempted to combine both frequency and density dependence into analyses of evolutionary dynamics, but in these few cases have either shown density (Bennington and Stratton 1998) or both factors (Levitan and Ferrell 2006; Mappes et al. 2008) to be important. Here I present evidence that directly measured instantaneous selection rates can (1) vary substantially with population dynamics and (2) are genotype by genotype dependent, findings which have implications for understanding the maintenance of diversity in natural populations and for using selection experiments to infer fitness.

Table 4.1. Two-way ANOVA statistics for frequency- and density-dependence of selection coefficients.

Focal clone	Competitor Clone	Factor	d.f.	Sum Squares	Mean Square	F	P
33_ROS03	351_EST02	Density	2	0.3955	0.1978	8.984	0.0014
		Frequency	2	0.1108	0.0554	2.517	0.1037
		Residuals	2	0.4843	0.0220		
47_BGN01	47_TMO01	Density	2	0.2115	0.1058	3.042	0.0682
		Frequency	2	1.8189	0.9094	26.159	<0.0001
		Residuals	22	0.7649	0.3048		
351_EST02	47_BOD02	Density	2	0.0249	0.0124	0.697	0.5111
		Frequency	2	0.0237	0.0119	0.664	0.5267
		Density*Frequency	4	0.2913	0.0728	4.080	0.0158
		Residuals	18	0.3213	0.0179		
44_PLY01	47_BGN01	Density	2	0.3337	0.1669	16.090	<0.0001
		Frequency	2	0.1439	0.0720	6.940	0.0058
		Density*Frequency	4	0.2694	0.0673	6.494	0.0020
		Residuals	18	0.1867	0.0104		
351_EST02	47_BGN01	Density	2	0.0070	0.0035	0.161	0.852
		Frequency	2	0.0612	0.0306	1.413	0.265
		Residuals	22	0.4767	0.0216		
351_FAR01	351_EST02	Density	2	0.0389	0.0194	0.767	0.476
		Frequency	2	0.0677	0.0339	1.336	0.283
		Residuals	22	0.5574	0.0253		

The interactions between ecological dynamics and natural selection have important consequences for evolutionary ecology (Reznick 2013). Density dependent selection was one of the first principles that unified the fields of evolution and ecology (Travis et al. 2013), as it represents one of the most fundamental feedbacks of ecology (*i.e.* population dynamics) on evolution (*i.e.* natural selection). As selection is a "change in genotypic frequency" (Endler 1986) and ultimately reflects the change in the ratio of densities of multiple competing

genotypes, it too is a component of ecological dynamics, just at a finer genotypic resolution than that of the population. Here I report the intriguing result that negative frequency dependence occurs more strongly at high population densities, whereas at lower densities it seems to be less prevalent. This was not universally the case (*i.e.* it was observed in 3 out of 6 strain pairs) but was found as a significant effect even taking into account data from all strain pairs. In addition, the effect of high density upon frequency dependant selection was similarly described by Levitan and Ferrell (2006) and Mappes et al. (2008). Interestingly, these two studies are the only two to have measured change in genotypic frequency, as opposed to another fecundity based fitness proxy (Bennington and Stratton 1998; Van Gossum et al. 2005).

Modelling studies by Eadie and Fryxell (1992) and Newton et al. (1998) provide additional evidence that frequency and density may interact in this way to influence selection. Together all of these results suggest a potentially consistent interaction between population density, genotypic frequency, and natural selection that could have fundamental implications in evolutionary ecology. The consequences of this finding are twofold: firstly that negative frequency dependent selection may be more important in populations that maintain high densities (*K*-selected). This is interesting as I then lack an obvious mechanism for maintenance of high diversity in *r*-selected species; for example, in bloom forming plankton. Second, for populations that fluctuate in density during growth cycles the added level of complexity in selection dynamics (*i.e.* interaction between frequency and density) would be expected to increase the maintenance of diversity, as negative frequency dependence operates more strongly at high density only once populations are established, as opposed to at low densities where population growth is less regulated.

I found a high diversity in the responses of selection to frequency and density between pairs of strains. Every pair provided a different response, except for the two pairs which showed no response, together comprising all possible combinations of frequency and density dependent selection: frequency only, density only, frequency and density dependence, and an interaction between frequency and density dependence. The complexity of responses shown by different pairs is novel and highlights the strain specific and highly complex nature of natural selection in diverse populations. To my knowledge, no experimental studies have investigated variation in selection responses (neither density nor frequency dependence) between many pairs of genotypes of the same species. Those that have investigated these responses have limited themselves to studying in detail the responses of selection (or other fitness proxies) between a pre-defined pair of phenotypes, designed in such a way that they expect to see frequency- and/or density-dependence. My results are in contrast to this, and present an independent test not just of the occurrence of interaction between frequency-and density-dependence on selection, but also of the prevalence of such interactions between multiple genotypes in a population.

The broad finding of ecological dependence on selection may also have technical implications in experimental evolution. The fact that selection coefficients are highly dependent on population dynamics (*i.e.* population density and genotype frequency), indicates that careful control of these dynamics during selection experiments is necessary to avoid inferences driven by the indirect effects of experimental manipulations (*i.e.* those that effect ecological dynamics). Two solutions to this problem exist: first, control of population density either continuously (*e.g.* in chemostats) or by equalising density between transfers as opposed to equalised volumes; second, short incubations for measurements of selection coefficients where population dynamics do not have time to vary substantially.

Chapter 5

Selection responses to experimental climate change in *Oxyrrhis marina*

5.0 Introduction

Climate change is expected to have a major impact upon the composition and function of marine ecosystems (Harley et al. 2006; Hoegh-Guldberg and Bruno 2010). To what extent natural populations have the potential to mitigate these impacts, through plasticity or adaptation via natural selection, is poorly-understood unknown (Reusch 2014). Marine microbes (i.e. bacteria and protists) underpin ecosystem function (Azam and Malfatti 2007): they are responsible for nearly all of oceanic primary production and around half globally (Longhurst et al. 1995), the majority of subsequent trophic transfer, and play major roles in global biogeochemical cycling (Arrigo 2005) being responsible for at least half of the Earth's carbon and nitrogen fixation. Due to their large population sizes, short generation times, and broad distributions (Fenchel and Finlay 2004; Vanormelingen et al. 2007; Watts et al. 2010) marine microbes are predicted to have high evolutionary potential. The implication is that marine microbial populations may be capable of mounting an appropriate response to climate change (Joint et al. 2011).

Experimental evidence that marine microbes can adapt to future climate change scenarios are still either rare or have considered limited evolutionary scenarios (Collins et al. 2014). Understanding the processes by which microbes will adapt is necessary if we are to make meaningful predictions on the composition and functioning of future microbial communities

in the sea. Thanks to their evolvability, studying evolution in action is possible with microbial species amenable to culture. In recent decades, experimental evolution, especially in prokaryotes, has revolutionised the study of evolutionary dynamics (Barrick and Lenski 2013; Buckling et al. 2009), and is equally suited to topics of applied interest such as adaptation to climate change (Latta 2008).

Selection requires genetic variation that, ultimately, is supplied into populations through mutation. Since mutations tend to be rare, larger populations tend to acquire new variation. Whilst some experimental studies have observed adaptation to new environments by selection on *de novo* mutations, typically in large populations (Collins and Bell 2004; Jin et al. 2013; Lohbeck et al. 2012; Pespeni et al. 2013), this overlooks the issue that most natural microbial populations already harbour considerable genetic variation (Godhe and Harnstrom 2010; Harnstrom et al. 2011; Kashtan et al. 2014; Lowe et al. 2005; Nagai et al. 2009; Penna et al. 2010) that can act as raw material for selection. Indeed, selection from standing variation (Barrett and Schluter 2008) is predicted to be an important component of adaptation to climate change, as species with broad distributions may already contain the variation necessary to adapt, thereby reducing the apparent reliance upon mutation supply, and enabling rapid contemporary adaptation (Bell and Collins 2008).

Attempts to infer evolutionary responses tend to base their findings on trait distributions that are predicted to relate to fitness (Clusella-Trullas et al. 2011; Etterson and Shaw 2001; Kremp et al. 2012) or make predictions about evolutionary potential based on population genetic parameters (Sunday et al. 2011). Whilst these approaches are appropriate for particular organisms where studying evolution *in situ* is impractical (e.g. due to long generation times or experimental intractability), a more detailed application of evolutionary concepts in marine ecology is necessary to assess the potential for evolutionary responses in

marine organisms (Collins 2010; Collins et al. 2014; Hoffmann and Sgro 2011; Munday et al. 2013; Reusch 2014; Reusch and Boyd 2013; Sunday et al. 2014). While empirical studies that have quantified intra-specific variation in eco-physiological responses to climate change (e.g. Chapter 2, (Kremp et al. 2012; Schaum et al. 2012) provide evidence for adaptive genetic variation within natural populations, there are few attempts to quantify the strength of selection (*i.e.* selection coefficients – (Chevin 2011)) from this variation (Lohbeck et al. 2012). The distinction is important as eco-physiological data *per se* may not reflect selection and hence fitness (Chapter 3): direct measures of selection may thus provide a better inference of selection from standing variation.

Many, if not most, low latitude species are shifting their ranges Polewards in response to climate warming (Parmesan and Yohe 2003; Sorte et al. 2010). It is therefore reasonable to assume that selection at any particular location may favour genotypes that are adapted to warmer environments. Predicting a response to elevated pCO_2 (i.e. acidification) is more difficult, but a reasonable assumption is that ocean acidification may favour genotypes from regions of higher pCO_2 variability, such as in coastal and eutrophic habitats or near hydrothermal vents (Beaufort et al. 2011; Hall-Spencer et al. 2008; Schaum et al. 2012). Moreover, levels of CO_2 vary on seasonal and daily timescales (Baumann et al. 2014), largely driven by biological processes such as respiration and photosynthesis; hence regions with higher biological activity have higher pCO_2 variability and organisms from productive coastal regions are expected to be more resilient to ocean acidification compared to those adapted to the open ocean (Duarte et al. 2013). It is important to note that the effects of climate change invoke multiple stressors simultaneously (Bopp et al. 2013), and there is a potential interaction between temperature and acidification (Harvey et al. 2013; Sett et al. 2014; Tatters et al. 2013), with eco-physiological data indicating that synergistic effects of combined stressors generate both negative and positive interactions effect different functional

groups and response types. Nonetheless, few evolutionary studies have yet to examine the interaction between temperature and pCO_2.

Widely distributed species, such as *Oxyrrhis*, which occupy a range of habitats over a large spatial scale may be locally adapted their natural habitats. Knowing how the seascape varies in term of these environmental parameters can inform us about how the population may be structured, and hence how it may respond to environmental change (Schmidt et al. 2008). Within the European North Atlantic, habitats experience a range of mean annual temperatures, which are highly correlated with latitude. One should then predict that under increased temperature regimes, selection should favour strains isolated from more southern (warmer) locations. For pCO_2 the picture is less clear as no clear gradient exists, but strains from areas of high productivity (e.g the Irish Sea) could be expected to be favoured under acidified conditions.

In the present chapter I quantify changes in selection coefficients between pairs of *Oxyrrhis marina* strains in response to elevated pCO_2 and temperature. I show that (1) both pCO_2 and temperature can affect natural selection in this species, (2) that predictions of local adaptation are better revealed by direct estimation of fitness as opposed to from eco-physiological rates, and (3) provide weak support for arguments suggesting strains from higher pCO_2 and temperature backgrounds will be favoured by natural selection under future climate change scenarios.

5.1 Methods & Materials

Experimental organisms and stock culturing

Selection responses were quantified by performing selection experiments between pairwise combinations of the following strains of *Oxyrrhis marina* Dujardin 1895, originally isolated from intertidal habitats on the North East Atlantic Ocean (Fig.1.1., Table 2.1.); 351_FAR03 (Faro, Portugal), 33_ROS03 (Roscoff, France), 44_PLY01 (Plymouth, UK), 44_WIR02 (Wirral, UK), 47_BGN01 (Bergen, Norway), 47_BOD01 (Bodo, Norway), & 47_TMO01 (Tromso, Norway). These strains were selected as they cover a broad range of temperatures, from a mean sea surface temperature of 6.6 °C in the North and 20.5 °C in the South and variation in mean annual pCO_2 from 319-402 µatm.

All strains were cultured in the laboratory at 16 °C and at a light intensity of ~80 µmol photons m^{-2} on a light:dark cycle of 14:10. Stock cultures were maintained in 32 PSU sterile filtered artificial sea water (SASW) enriched with f/2 (Sigma Aldrich, UK) and fed with *Dunaliella primolecta* at a cell density of approximately 3×10^5 cells ml^{-1}. Stock cultures were sub-cultured once per month, and prior to experiments had been maintained in this state for between 2 and 5 years. At least one month before experiments, stock cultures were sub-cultured in SASW without f/2 and fed with heat killed *Escherichia coli* (Lowe et al. 2010b) at a density of 1.25-2.5 × 10^6 CFU ml^{-1} to deplete live food. Fresh *E. coli* was added to these pre-experimental cultures every 2-5 days, in addition to combinations of antibiotics (Penicillin/Streptomycin & Gentamycin) to prevent bacterial growth.

Selection experiments

I performed selection experiments on the following seven pairs of strains: 44_PLY01 & 47_BGN01, 351_FAR03 & 47_BOD02, 47_BGN01 & 47_TMO01, 47_BOD02 & 44_WIR01, 47_TMO01 & 44_PLY01, 33_ROS03 & 47_BGN01, 47_TMO01 & 351_FAR01. Selection experiments were performed in 500 mL microcosms, initially containing 250 mL SASW, and inoculated with heat killed *E. coli* ($1.25\text{-}2.5 \times 10^6$ CFU mL^{-1}) and *Oxyrrhis marina* at an initial total population density of ~1000 cells mL^{-1} and at an equal ratio (*i.e.* ~500 mL^{-1} for each strain). All microcosms were inoculated independently by dilution of stock cultures to 2500 cells mL^{-1} and addition of 50 mL of diluted stock for each strain to 150 mL fresh SASW with added heat killed *E. coli*. Microcosms were randomly assigned to one of four treatment groups, temperature low (16 °C) and high (20 °C) and pCO_2 high (374 µatm) or low (1180 µatm) in a full factorial design and in replicate (n=3), roughly in line with IPCC estimates of environmental change up until the year 2100. Microcosms were manipulated for temperature using water baths, and pCO_2 was manipulated by bubbling of pre-mixed Air/CO_2 supplied from gas cylinders (BOC Special Products, UK) via fine pulled glass Pasteur pipettes. To estimate the densities of each strain during the course of the experiments, the microcosms were sampled at 0 and 48 hours by removing 50 mL of culture after gentle mixing and immediate centrifugation at 4600 rpm and 4 °C for 30 minutes. Supernatant was removed and pellets were immediately frozen at -20 °C for up to 3 days prior to DNA extraction. While this experimental time period represents about 2 generations in *Oxyrrhis marina*, other work has indicated that short-term responses can be representative of long-term responses to environmental challenges (Tatters et al. 2013).

DNA extraction and quantitative genotyping

I used quantitative microsatellite genotyping typing (Chapter 2) to determine strain frequencies for pairs of strains. Frozen DNA pellets were defrosted and washed twice in PBS. Genomic DNA was extracted from the cell pellet by adding 100 µL Chelex-100™ (Bio-Rad Laboratories) solution (5% w/v) and then incubating the samples at 95°C for 1 hour. Microsatellite loci were amplified by polymerase chain reaction in replicate (n=4) using one of three microsatellite primer pairs for each combination of strains (see Chapter 3). PCR reactions contained 2 µL of the Chelex-extracts as a template DNA in 10 µl reaction volumes that contained Green GoTaq® reaction buffer (pH 8.5) (Promega), 10 µg BSA, 0.3 pmol of forward primer (5' labelled with either 6-FAM, NED, PET or VIC, Applied Biosystems), and 0.3 pmol reverse primer. Thermal cycling conditions were: 95°C for 1 min, N_c×[95°C 30 s, 58°C 45 s, 72°C 45 s], 72°C 10 min, where N_c represents the number of cycles (see Chapter 2). PCR products were pooled with Genescan 500 liz size standard (Applied Biosystems) and separated by electrophoresis on an AB3130xl (Applied Biosystems).

Data analysis

Microsatellite alleles were scored using Genemapper v.3.0 (Applied Biosystems) and peak heights were measured for each allele belonging to the strains used in each selection experiment. The peak height ratio was log-transformed and for each sample a strain ratio was calculated using a strain pair specific calibration curve (sees Chapter 2). Selection coefficients (*s*) for a target strain versus a non-target strain were estimated from the slope of natural log target strain ratio with time.

A global analysis of selection coefficients was performed with a linear mixed effects model, with selection coefficient as the response variable, temperature and pCO_2 as fixed effects, and

strain pair as a random effect. Prior to analysis, all strain pairs were aligned so that the strain with the mean positive selection coefficient across treatments was the focal strain. Due to high variation in responses between pairs of strains I analysed selection coefficients for each pair independently using a two-way ANOVA, with temperature and pCO_2 as factors.

For comparison with environmental data (collected as in Chapter 2), I calculated a response variable for selection of both temperature and pCO_2 termed the "pCO_2/temperature selection response" for each strain pair by subtracting the mean selection coefficient at low temperature/pCO_2 from the mean selection coefficient at high temperature/pCO_2. Difference in environmental parameters (as in Chapter 2) was also calculated for each pair of strains, by subtracting values for non-focal strain from those of the focal strain. I analysed relationships between the pCO_2/temperature selection responses and difference in environmental parameters by linear least-squares regression. All statistical analyses were performed using R v3.1.0 (R Core Development Team) and all data are presented as ± 1 SE.

5.2 Results

Both pCO_2 and temperature have significant effects on selection coefficients across my pairs of strains (Mixed effects model, χ^2_2=8.02, P<0.05). Of these two stressors, pCO_2 (-0.032 ± 0.013) had a greater mean effect size than did temperature (-0.005 ± 0.003). There was no evidence of an interaction, suggesting the effects of pCO_2 and temperature on selection are not synergistic for *O. marina*. Individual analyses of the seven strain pairs, revealed only three significant responses to pCO_2 or temperature: for 351_FAR03& 47_BOD02 there was a significant interaction between pCO_2 and

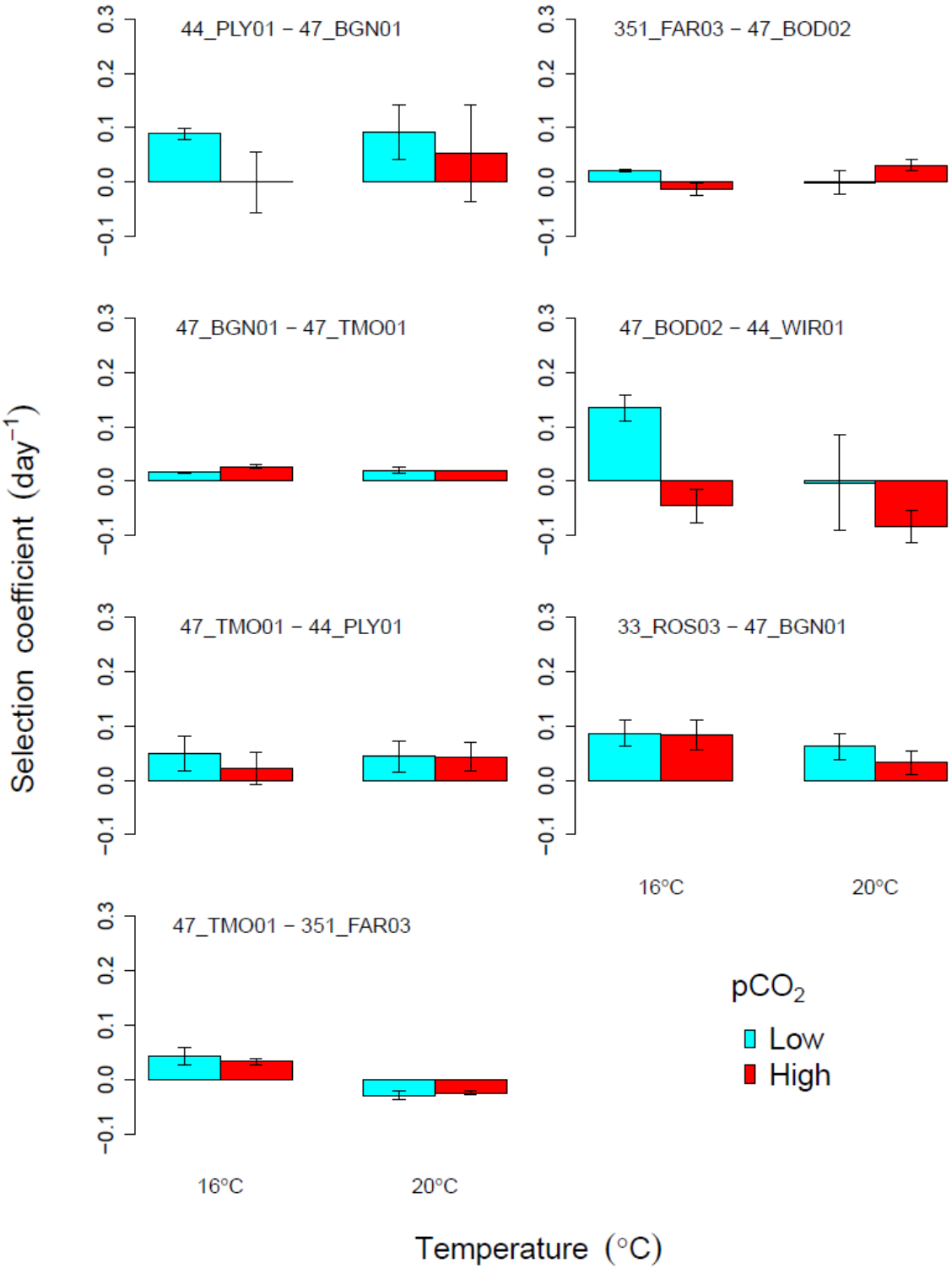

Fig.5.1. Selection coefficients of seven strains of *Oxyrrhis* in response to pCO_2 and temperature treatments. Strains are indicated in the top left of each panel, the first given is the target strain; the second is its competitor. Errors bars indicate ± 1 SE.

temperature (ANOVA, $F_{1,8}=6.099$, $P<0.05$) with 351_FAR03 responding negatively to increased pCO_2 at 16 °C and positively to pCO_2 at 20 °C relative to 47_BOD02. For

47_BOD02 & 44_WIR02 there was a significant effect of pCO_2 (ANOVA, $F_{1,10}=5.56$, $P<0.05$) with 44_WIR02 increasing in fitness relative to 47_BOD02 under elevated pCO_2 levels. I found a significant effect of temperature for 47_TMO01 & 351_FAR01 (ANOVA, $F_{1,10}=49.10$, $P<0.001$), with 351_FAR01 increasing and 47_TMO01 decreasing in fitness at 20 °C (Fig.5.1.).

To test local adaptation I compared responses with environmental backgrounds. There were no significant associations between the temperature selection response and difference in annual SST, spring SST, SST variability, or sampling month SST, though in all cases there was a weak positive correlation present in the data (Fig. 5.3.), suggesting a weak association between local adaptation and selection in response to elevated temperature. For the pCO_2 selection response there was a significant positive association with difference in sampling month fCO_2 (Regression, $R^2=0.588$, $P<0.05$) suggesting strains isolated from periods of seasonal pCO_2 elevation are favoured by selection in response to ocean acidification. Relationships between pCO_2 selection rates and all other environmental parameters were non-significant, but as with temperature all provided evidence of a weak positive association (Fig.5.2.) with selection responses potentially favouring strains from higher environmental fCO_2 or greater variation in fCO_2.

5.3 Discussion

Understanding how evolutionary processes will unfold for marine organisms in response to climate change has important consequences for marine ecosystem

Table 5.1. Two-way ANOVA statistics for selection responses to temperature and *p*CO_2.

Target strain	Competitor	Treatment	d.f.	sumsq	mean sq	F	P
44_PLY01	47_BGN01	CO_2	1	0.01229	0.01229	1.314	0.281
		Temp	1	0.00245	0.00245	0.262	0.621
		Residuals	9	0.08415	0.00935		
351_FAR03	47_BOD02	CO_2	1	2E-06	2E-06	0.004	0.9533
		Temp	1	0.00037	0.00037	0.683	0.4326
		CO_2*Temp	1	0.00329	0.00329	6.099	0.0387
		Residuals	8	0.00431	0.00054		
47_BGN01	47_TMO01	CO_2	1	6.3E-05	6.32E-05	1.647	0.231
		Temp	1	1E-05	1.03E-05	0.268	0.617
		Residuals	9	0.00035	3.84E-05		
47_BOD02	44_WIR02	CO_2	1	0.05096	0.05096	6.67	0.0296
		Temp	1	0.02296	0.02296	3.005	0.1171
		Residuals	9	0.06877	0.00764		
47_TMO01	44_PLY01	CO_2	1	0.00059	0.00059	0.247	0.631
		Temp	1	0.00021	0.00021	0.087	0.774
		Residuals	9	0.02133	0.00237		
33_ROS03	47_BGN01	CO_2	1	0.00059	0.00059	0.247	0.631
		Temp	1	0.00021	0.00021	0.087	0.774
		Residuals	9	0.02133	0.00237		
47_TMO01	351_FAR03	CO_2	1	2.9E-05	2.9E-05	0.102	0.757
		Temp	1	0.01267	0.01267	44.644	<0.0001
		Residuals	9	0.00255	0.00028		

conservation (Hoffmann and Sgro 2011; Parmesan 2006; Reusch 2014). Species with wide distributions and high genetic diversity may adapt by natural selection from standing variation (Barrett and Schluter 2008), and so predicting which genotypes or populations are best pre-adapted to future conditions could be useful for targeting conservation efforts (Pauls et al. 2013; Sgrò et al. 2011). Here I provide evidence that strains sampled from regions of

higher absolute pCO_2 may be favoured under ocean acidification scenarios, strains from higher temperatures may be selected by global warming, and that these two stressors do not interact to impact selection. In Chapter 2 I highlighted that eco-physiological rates were not indicative of fitness in *Oxyrrhis marina*, so direct measures of competitive fitness (*i.e.* selection coefficients) may provide better inference into assessments of which genotypes will be selected under future environmental change scenarios. In this chapter I show that selection coefficients are indeed more predictive of a strain's environmental background, and that compared to eco-physiological data may provide better estimates of future natural selection from diverse and widespread populations.

Selection coefficients measure fitness directly by estimating the rate of change in frequency of a genotype relative to another (*i.e.* the rest of the population) under competition (Chevin 2011). For assessing changes in competitive fitness under simulated climate change conditions, the direction of selection is relatively unimportant but changes in magnitude of selection can be inferred as those experimental conditions (here increased temperature or pCO_2) favouring one strain, in terms of natural selection, compared to another. I found evidence of both increased temperature and pCO_2 changing selection rates among my strains, which suggests that both marine climate change impacts could alter the course of evolution in this species. Interestingly, these data suggest that pCO_2 may be more important than temperature in driving selection, as the mean effect size of pCO_2 was greater than for temperature. This is not consistent with the few other studies on marine microbes to have considered the effects of both stressors simultaneously (Tatters et al. 2013) or assessed the drivers of global distributions in natural samples (Flombaum et al. 2013; Swan et al. 2013). Moreover, the lack of interaction between pCO_2 and temperature on selection in *Oxyrrhis* contrasts with the idea that synergistic interactions will impact growth, photosynthesis, and calcification in a range of marine organisms (Harvey et al. 2013; Sett et al. 2014; Tatters et al.

2013). If confirmed in other studies, the implication is a disparity between evolutionary and ecological studies of climate impacts.

Response to stressors is genotype dependant, but maybe not random. Thus, for individual strain pairs the significant changes in selection, where they occur, make sense in terms of local adaptation of strains. For example, under increased temperature 351_FAR03 increased in fitness relative to 47_TMO01, which should be expected since 351_FAR03 is isolated from an environment with the highest mean temperature, and 47_TMO01 the lowest. Similarly, 44_WIR02 comes from the environment with the highest fCO_2 and intra-annual fCO_2 variability, and increased in fitness at high pCO_2 relative to 47_BOD02 which came from an environment with comparatively low fCO_2 and fCO_2 variability. The interaction between 351_FAR03 & 47_BOD02 may be explained by each strain being more negatively impacted by high pCO_2 at their more unnatural temperatures, 16 °C for 351_FAR03 and 20 °C for 47_BOD02. These specific cases support other empirical evidence that populations from low latitudes should be favoured under elevated temperatures (Thomas et al. 2012) and that populations from seas with higher pCO_2 variability may be favoured under ocean acidification (Beaufort et al. 2011; Schaum et al. 2012).

Local environment has proved to be an important determinant of the response to environmental conditions in terrestrial and freshwater taxa (De Block et al. 2013; Mitchell and Lampert 2000; Nilsson-Ortman et al. 2012; Van Doorslaer et al. 2009; Yampolsky et al. 2014). Changes in selection could be explained by local adaptation of strains which inhabit a wide range of environmental conditions across the European Atlantic continental shelf seas. That there was only one significant association between

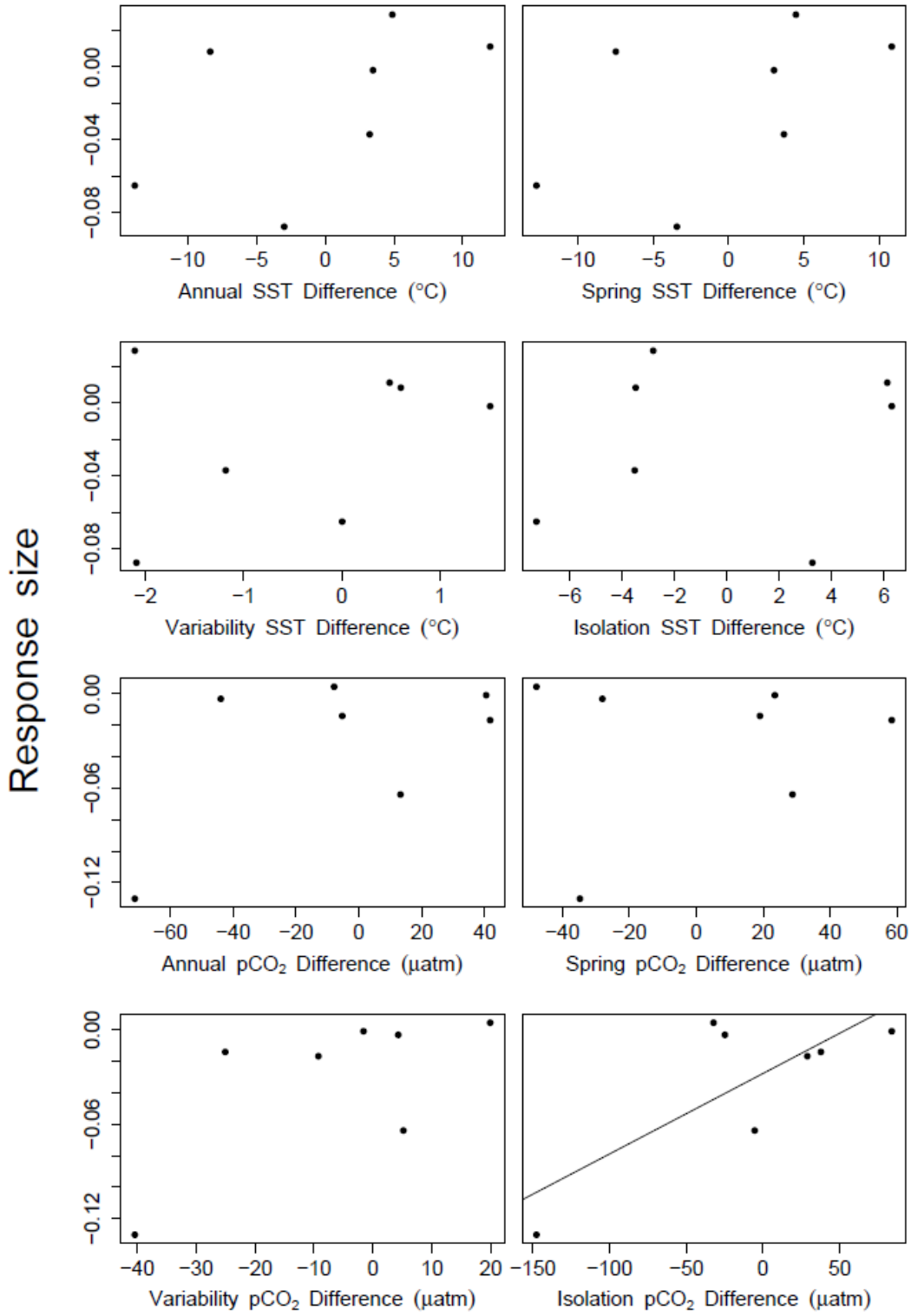

Fig.5.2. Relationship between temperature- and pCO_2-selection responses of North Atlantic strains of *Oxyrrhis* and differences between estimated environmental parameters from which strains were isolated. Each point represents one strain pair.

the measured selection responses and environmental background of strains tested (for fCO_2 monthly measurements – see Fig.5.2.) which to some extent indicates that strains which have experienced a history of higher pCO_2 may be pre-adapted to future ocean acidification, and have a selective advantage relative to those strains which are typically found in regions of lower pCO_2. This is interesting as approaches to forecasting species responses to climate change can emphasise functional traits or phylogeny (Buckley and Kingsolver 2012) rather than intraspecific variability and the local environment. Moreover, the rather few studies that quantify the potential for adaptation to ocean acidification mean that identifying robust patterns (e.g. related to taxonomy, location) is not yet possible (Kelly et al. 2013). Studies in which isolates are truly planktonic, and hence for which more complete environmental data are available, show environmental background to be an important predictor of response to elevated temperature (Thomas et al. 2012) and pCO_2 (Beaufort et al. 2011; Schaum et al. 2012). My selection response data support this assertion, though only weakly, and for *Oxyrrhis* suggest that absolute levels of pCO_2, rather than variability, are most predictive of this response. Thus genotypes adapted to more eutrophic habitats, especially those in productive coastal seas, areas of high nutrient loading, and temporally variable habitats may be more resilient to increased pCO_2 and therefore represent and pool of genes that could facilitate adaptation. Evidence for local adaptation is emerging in some marine invertebrates (Sanford and Kelly 2011), but still is rare in microbial populations where large population sizes, high dispersal capacity and use of clonal reproduction presumably limits the capacity for response to local conditions . Given my low sample size (n=7) and that my environmental estimates of fCO_2 and temperature are mostly taken from offshore sampling sites (see also Schmidt et al. (2008) for approach), rather than the actual intertidal habitats where *Oxyrrhis* is isolated from, possible explanations for the lack of association could be that my environmental data do not reflect well enough the true environments of my strains

(Yampolsky et al. 2014) and there is a lack of statistical power (see e.g. variability in the response described by Thomas et al. (2012)). A second issue is the high level of genetic diversity within local microbial populations (Lowe et al. 2010c). Moreover, like other studies, I only quantified the response of one strain per site (Thomas et al. 2012); however, given that intraspecific variability is likely important for species persistence (Menden-Deuer and Rowlett 2014), by analogy a lack of sampling within sites may not capture the overall response of a population to climate change (see also Davis et al. (1998a); Davis et al. (1998b) for importance of considering intraspecific variation when predicting responses to climate change).

Despite the lack of significant associations, especially for temperature responses, there were weak positive correlations between environmental parameters and selection responses, which is in contrast to eco-physiological (growth rate) responses (Chapter 1) where all weak correlations were negative, indicating strains isolated from colder and less acidic seas responded more positively to increased temperature and pCO_2 respectively. This could be interpreted as evidence that selection coefficients provide more astute predictions, in terms of fitness, than eco-physiological responses when assessing the evolutionary response of intra-specific diversity to climate change. Given that growth rates are likely to be poor predictors of fitness (Chapter 2) and that adaptation to climate change is an evolutionary, as opposed to a physiological, challenge, it is suggested that predicting evolutionary responses of diverse populations may be best tackled using selection experiments and inferring fitness directly under competition by estimating selection coefficients.

Chapter 6

General Discussion

The data presented in this thesis reveal the importance of intra-specific variation in predicting species' responses to climate change. I have shown that for *Oxyrrhis*, a widely distributed, abundant, heterotrophic dinoflagellate, that substantial eco-physiological variation in response to experimentally simulated climate change (increased pCO_2 and temperature) exists between strains, and that this variation is not predicted by local adaptation of strains to prevailing environmental conditions (Chapter 2). In order to study strain specific dynamics in mixed populations, I developed a novel genotyping method utilising high polymorphism in microsatellite loci, and used this to show that growth rates do not predict fitness between pairs of strains in competition experiments (Chapter 3). I then used the quantitative microsatellite genotyping to investigate frequency- and density- dependent selection between many pairs of strains, and found that, whilst not ubiquitous, a prevalent interaction exists between population density and genotype frequency, whereby negative frequency-dependent selection is intensified at higher population densities (Chapter 4). Finally, we examined the response of selection, between multiple pairs of *Oxyrrhis* strains, to experimentally simulated climate change (Chapter 5). These data show that selection is significantly affected by both increased pCO_2 and temperature, and that the selection responses fit the model of local adaptation better than do the eco-physiological responses, suggesting that selection coefficients could be employed to predict natural selection from standing variation in natural populations.

Advances in understanding biological responses to climate change require greater theoretical and empirical integration of ecological and evolutionary processes. In Chapter 4 I demonstrated an interaction between genotype frequency and population density which

revealed that negative frequency dependent selection, a major force in maintaining diversity, is stronger at higher population densities and either weak or absent at lower population densities. This interaction between frequency- and density- dependent selection has been rarely explicitly tested by experiments, but in those rare cases supports our finding (Levitan and Ferrell 2006; Mappes et al. 2008). Interestingly, experimental evolution studies using the bacterium *Pseudomonas fluorescens* reveal an effect of population density on the evolution of genotypic diversity. *P. fluorescens* undergoes an adaptive radiation in laboratory microcosms into three ecologically and genetically distinct morphs, which are maintained by negative frequency dependent selection. Reduced population densities either by explicitly manipulating density, or by employing either predators (Meyer and Kassen 2007) or bacteriophages (Buckling and Rainey 2002) as natural enemies to lower the population densities, led to weakening of negative frequency dependent selection and reduced diversification. Together this suggests that the interaction between frequency- and density-dependent selection may be general and that the mechanism for this is stronger intra-specific competition intensifying negative frequency dependent natural selection. Thus, this pattern may be a widespread pattern occurring in nature, and warranting both further investigation and incorporation into models of eco-evolutionary dynamics.

The presence of high levels of intra-specific variation may be an endemic feature of marine microbial species (Kashtan et al. 2014; Lowe et al. 2005; Lowe et al. 2010c). This presents marine ecologists with a substantial challenge, both to understand its role in ecosystem functioning, and to incorporate how it will respond to changing climatological and oceanographic conditions. Most assessments of biological responses to climate change use one or few isolates to represent a species (Crawfurd et al. 2011; Dixson et al. 2010; Doo et al. 2011; Fu et al. 2008; Widdicombe and Needham 2007), or in some cases entire functional groups of micro-organisms (Cripps et al. 2014; Hutchins et al. 2013). Clearly, if microbial

populations are so diverse in their strain specific responses, this approach could lead us to make incorrect inferences about how microbial species may respond to environmental change. It is suggested that as a standard procedure for assessing species' responses to climate change, genotypes from a diverse set of sampling environments across the species' distribution range are examined, to explicitly quantify the levels of phenotypic variation in responses. There has been an increasing trend to do just this in recent years (Kremp et al. 2012; Schaum et al. 2012), however many recent publications still persist in claiming broad effects, either for species or higher taxonomic or functional groups of organism, based on one or few isolates of one or few species (Cripps et al. 2014; Hutchins et al. 2013). Examining intra-specific variation does of course represent a significant challenge, especially for those studying organisms less amenable to laboratory experimentation, but to achieve credibility and confidence in results, and to make accurate predictions of species climate change responses, is absolutely necessary.

The investigation of evolutionary rescue (ER) has advanced considerably in recent years and provides a number of insights into both the processes leading to, and consequences of evolutionary adaptation to environmental change (Gonzalez et al. 2013). The key early predictions of ER theory (Bell & Collins 2008) have all found empirical support and indicate that larger population size (Bell & Gonzalez 2009; Samani & Bell 2010), higher genetic variation (Lachapelle and Bell, 2012; Ramsayer et al. 2013; Stelkens et al. 2014), and decreased speed of environmental change (Bell & Gonzalez, 2011; Collins & de Meaux 2009), should all lead to a greater probability of ER. For mutation-limited populations, with relatively small effective population sizes and long generation times (*e.g.* animals), standing variation is a crucial factor in adaptation to environmental change, although it has been argued that this may not be the case for microbes, such as bacteria and protists (Bell 2013). However, recent research has highlighted that effective population sizes of protists may be

significantly smaller than we would expect based on their very large population sizes (Watts et al. 2013). Thus, at least for some microbes, the mutation supply may not be that high, which would shift the emphasis for adaptation onto standing variation.

In asexual clonal organisms high standing genetic variation could pose a potential barrier to adaptation, due to clonal interference (Lanfear et al. 2014). Clonal interference may occur where beneficial mutations arise in different genetic backgrounds and compete with each other, impeding their fixation in a population (Orr 2010). Despite this issue, standing variation is still likely to play an important role in adaptation to climate change in many microbial populations. Recombination either by horizontal gene transfer (Keeling and Palmer, 2008; McDaniel et al. 2010) or meiotic sexual reproduction is prevalent in the microbial world. Many protists are known to be sexual (Raikov 1995) and many more, such as *Oxyrrhis*, are likely to be although this is unknown at present (Lee et al. 2014). So even if distinct beneficial mutations arise in different individuals they can feasibly be united by recombination.

I have shown that eco-physiological rate processes (i.e. growth rates) are poor predictors of fitness, even in an organism that grow by binary fission and over relatively short timescales (~ 2 generations). This finding has implications for how we assess species responses to climate change. The appropriate response, especially for micro-organisms, to climate change that should be quantified is that of fitness, as responding to relatively slow changes in climate is an evolutionary, rather than an ecological or physiological challenge. Imagine we took some Bushmen, the indigenous people of the Kalahari desert, and transplanted them to the Arctic Circle. They would show some level of decrease in performance, analogous to a negative eco-physiological response, and most likely would be unable to survive. Clearly this does not mean humans cannot survive in the Arctic Circle, as the Inuit peoples' persistent

inhabitation of that region testifies. Though this analogy may sound ridiculous, it is almost exactly this approach that eco-physiological responses to climate change employ. Given this, it is suggested that rather than quantifying eco-physiological rate processes, a more appropriate measure would be an evolutionary rate process, such as selection coefficients.

Research examining biological responses to ocean acidification has tended to focus on either autotrophic or calcifying organisms that are expected, for physiological reasons, to either increase or decrease in performance under elevated pCO_2 scenarios (Ridgwell and Schmidt 2010; Ries et al. 2009). It is unclear how species which are neither autotrophic nor calcifying will respond, with most studies finding little or no effect of acidification. Here I have shown that for *Oxyrrhis* there are subtle yet significant effects of ocean acidification in some strains, both on growth and cell size, but more importantly that elevated pCO_2 has a significant effect upon selection rates. This finding is important, as it shows that pCO_2 is likely to be an important evolutionary driver, even for non-calcifying heterotrophic organisms. Joint et al. (2011) hypothesised that ocean acidification would not affect marine microbes, and though they likely meant "affect" in a somewhat negative context, my data indicate that they may be "affected" in that pCO_2 can change selection between genotypes, and hence alter the course of future evolution in microbial species. Broadly speaking, I would tend to agree with Joint et al. (2011), and my selection data supports this assertion by showing that (1) adaptive variation to increased pCO_2 exists in *Oxyrrhis* and (2) that this variation is subject to selection by ocean acidification experiments, and could be predicted by geographic variation in pCO_2. It remains to be seen whether this holds in a wider range of organisms, but by following the selection from standing variation approach outlined in this thesis, in conjunction with other experimental, observational, and theoretical approaches, the ocean acidification research community can work towards a greater understanding of biological responses to ocean acidification.

Appendix 1.

Carbonate system parameters

Here are estimated carbonate system parameters for the experimental treatments used in this thesis (Table A1.1). These values were obtained by total alkalinity (A_T) measurements, in triplicate, of three batches of SASW at the UKOACF at the National Oceanography Centre, Southampton, and using the values of pCO_2 from gases of known partial pressures supplied by BOC (Special Products, UK). Values are given for mean, maximum and minimum A_T values, where maximum and minimum represent the mean ± 1 standard deviation.

Table A1.1. Carbonate system parameters for experimental manipulations used in this thesis.

	Salinity (PSU)	Temperature (°C)	pCO_2 (µatm)	pH	CO_2 (mol/kg)	fCO_2 (µatm)	HCO_3 (mol/kg)	CO_3 (mol/kg)	DIC (mol/kg)	A_T (mol/kg)
Mean	32	16	379	8.092	1.40E-05	378	0.001975	0.000176	0.002164	0.002404
	32	16	741	7.837	2.73E-05	738	0.002145	0.000106	0.002278	0.002404
	32	16	1123	7.671	4.14E-05	1119	0.002221	0.000075	0.002337	0.002404
	32	20	379	8.091	1.24E-05	378	0.001921	0.000198	0.002132	0.002404
	32	20	741	7.840	2.43E-05	739	0.002107	0.000122	0.002253	0.002404
	32	20	1123	7.677	3.69E-05	1119	0.002192	0.000087	0.002316	0.002404
Maximum	32	16	379	8.108	1.40E-05	378	0.002048	0.000189	0.002250	0.002506
	32	16	741	7.853	2.73E-05	738	0.002228	0.000114	0.002370	0.002506
	32	16	1123	7.688	4.14E-05	1119	0.002309	0.000081	0.002431	0.002506
	32	20	379	8.107	1.24E-05	378	0.001991	0.000213	0.002216	0.002506
	32	20	741	7.857	2.43E-05	739	0.002188	0.000132	0.002343	0.002506
	32	20	1123	7.694	3.69E-05	1119	0.002278	0.000094	0.002409	0.002506
Minimum	32	16	379	8.076	1.40E-05	378	0.001901	0.000163	0.002078	0.002302
	32	16	741	7.819	2.73E-05	738	0.002061	0.000098	0.002186	0.002302
	32	16	1123	7.654	4.14E-05	1119	0.002132	0.000069	0.002243	0.002302
	32	20	379	8.075	1.24E-05	378	0.001850	0.000184	0.002047	0.002302
	32	20	741	7.823	2.43E-05	739	0.002025	0.000113	0.002163	0.002302
	32	20	1123	7.660	3.69E-05	1119	0.002105	0.000080	0.002222	0.002302

Appendix 2.

Cell counting technique

This appendix outlines methods used to count cells and measure their area in Chapter 2. Cultures were agitated by shaking and 10 mL was fixed with 2 % v/v Lugol's iodine directly into 10 mL Uhtermol Chambers. Samples were allowed to settle for at least 20 hours prior to imaging. Cells settled onto the cover slip thickness bases of Uhtermol Chambers and were Imaged using a Zeiss Axiovert A.1 inverted microscope under DIC at 50 × magnification. For each sample, five images (e.g. Fig.A2.1) were taken with a Spot Insight 2.0 mp monochrome camera (Spot Imaging Solutions, USA), cell counts and mean cell areas were calculated with ImageJ (National Institutes of Health, USA) using the macro provided below (Fig.A1.2).

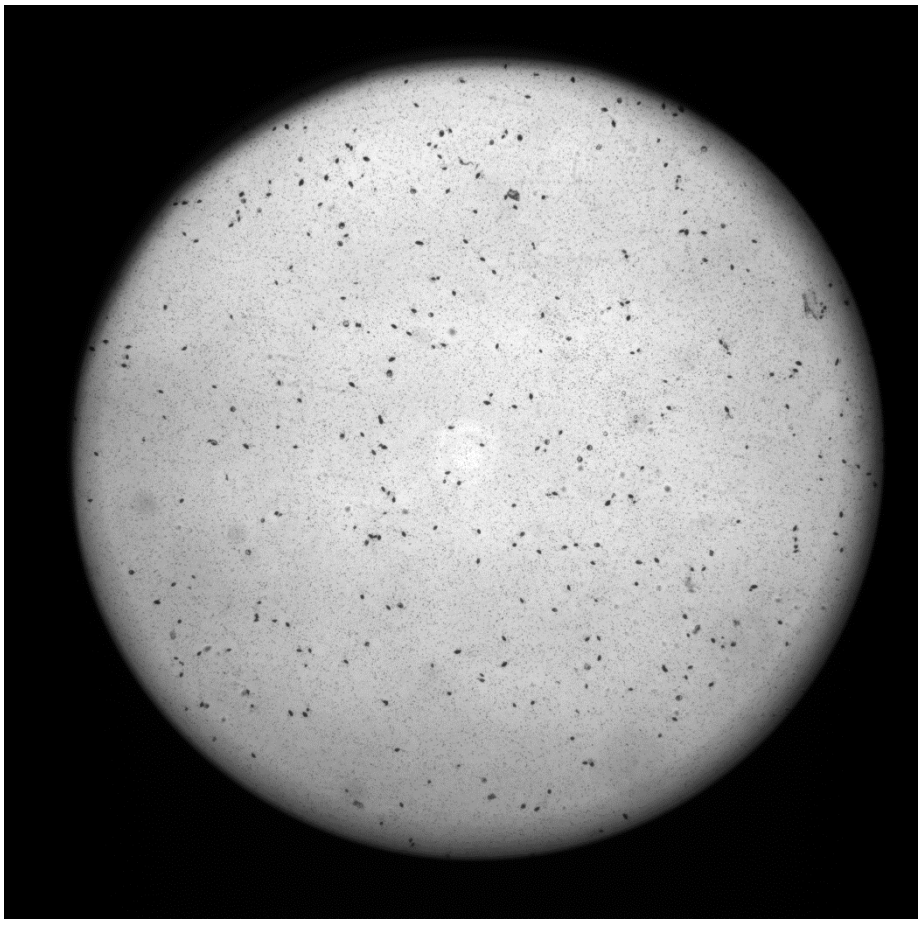

Fig.A1.1. Example image used for automated image analysis for cell counts and cell area measurements.

Cell areas were calculated by converting the number of pixels into μm^2 using the conversion factor of 0.43 pixels μm^{-2}.

```
for (i = 0; i < n; i+=1){


run("Specify...", "width=1300 height=1300 x=1150
y=900 oval centered");
List.setMeasurements;
mode=List.getValue("Mode");
min=List.getValue("Min");
   t1 = min;
   t2 = min+((mode-min)/2.15);
//run("Threshold...");
setThreshold(t1, t2);
run("Convert to Mask");
run("Watershed");
run("Specify...", "width=1300 height=1300 x=1150
y=900 oval centered");
run("Analyze Particles...", "size=20-130
circularity=0.65-0.99 show=Nothing display
summarize");
run("Open Next");


}
```

Fig.A2.2. Macro used in automated image analysis using ImageJ, where n is the number of photos to be analysed in a folder.

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