GENOTYPE-BY-TEMPERATURE INTERACTIONS MAY HELP TO MAINTAIN CLONAL DIVERSITY IN ASTERIONELLA FORMOSA (BACILLARIOPHYCEAE)[1]

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Abstract

Marine and freshwater phytoplankton populations often show large clonal diversity, which is in disagreement with clonal selection of the most vigorous genotype(s). Temporal fluctuation in selection pressures in variable environments is a leading explanation for maintenance of such genetic diversity. To test the influence of temperature as a selection force in continually (seasonally) changing aquatic systems we carried out reaction norms experiments on co-occurring clonal genotypes of a ubiquitous diatom species, *Asterionella formosa* Hassall, across an environmentally relevant range of temperatures. We report within population genetic diversity and extensive diversity in genotype specific reaction norms in growth rates and cell size traits. Our results showed genotype by environment (GxE) interactions, indicating that no genotype could outgrow all others across all temperature-environments. Subsequently, we constructed a model to simulate the relative proportion of each genotype in a hypothetical population based on genotype and temperature specific population growth rates. This model was run with different seasonal temperature patterns. Our modelling exercise showed a succession of two to several genotypes becoming numerically dominant depending on the underlying temperature pattern. The results suggest that (temperature) context dependent fitness may contribute to the maintenance of genetic diversity in isolated populations of clonally reproducing microorganisms in temporally variable environments.

Keywords

Coexistence, environmental variability, fluctuating selection, similarity, thermal reaction norms

Abbreviations

GxE genotype by environment

AFLP amplified fragment length polymorphism

SA/V surface to volume ratio

Introduction

How genotypic diversity is maintained within populations remains a central question in ecology (Rainey et al. 2000). In plankton ecology this is sometimes referred to as the "second paradox of the plankton" (Hebert and Crease 1980): how can so many different clones of phytoplankton taxa co-occur within the seemingly unstructured environment of a well mixed lake? In asexual organisms, clonal selection is expected to favour the most vigorous genotype, thereby eroding the genetic diversity in isolated populations (Bell 1991, Vorburger and Ramsauer 2008). However, many phytoplankton species, including diatoms show large genetic diversity within and among populations (Medlin et al. 2000). Temporal fluctuations in selection pressure on genotypes in seasonally changing environments is one of the leading explanations for maintenance of genetic diversity, both in theoretical (Bell 1982, Gillespie and Turelli 1989, Maynard Smith and Hoekstra 1980, Tilman 1982) and empirical studies (Bell 1991, Brand et al. 1981, Mitchell et al. 2004, Rynearson and Armbrust 2000).

Variability in environments may modify and diversify the expressed phenotype of any given genotype (Via and Lande 1985). Phenotypic plasticity, the ability of a single genotype to express a discrete or continuous range of phenotypes across different environments (Stearns 1989) may work as an adaptive strategy to cope with a range of environments. It can therefore mask genetic diversity (De Jong 1990) and delay competitive exclusion (Sebens and Thorne 1985). The profile of phenotypes produced by a genotype across environmental gradients is

known as a reaction norm (Via and Lande 1985). Reaction norm experiments are a classic way to illustrate phenotypic plasticity (Wolterek 1909) and to test for the occurrence of genotype by environment (GxE) interactions. Such experiments have been used for a wide array of species, both in animals (e.g. *Daphnia magna* (Giebelhausen and Lampert 2001), *Drosophila melanogaster* (David et al. 1994), *Bicyclus anynana* (Holloway and Brakefield 1995)) and in plants (e.g. *Potentilla glandulosa* and *Achillea millefolium* (Clausen et al. 1941)). A GxE interaction occurs when the mean trait value of a genotype varies across environments and the ranking order of genotypes, in respect to their mean trait value, varies among environments (or more graphically, if genotype specific reaction norm slopes are non-parallel or even cross) (De Jong 1990). Hence GxE interactions are an expression of the extent to which environmental variability promotes diversity at the intraspecific level (Pigliucci 2005). Genotype by temperature interactions are of specific interest, since temperature is an all-pervasive environmental factor affecting biochemical, physiological and behavioural processes at all levels ((Mitchell et al. 2005) and references therein).

The extent of genetic diversity within and among populations of freshwater diatoms has been documented in a series of studies. Random amplification of polymorphic DNA (RAPD) analysis of *Fragilaria capucina* Desmazières clones isolated along a gradient of North American lakes showed genetic variability among populations (Lewis et al. 1997) as did a microsatellite marker study on *Sellaphora capitata* on six populations in Europe and Australia with genotypes clearly clustering according to their geographic origin (Evans et al. 2009). The same picture emerged also in an amplified fragment length polymorphism (AFLP) analysis of *Asterionella formosa* populations of geographically close Dutch lakes that showed high within and among population genetic diversity with clear clustering of genotypes according to lake of origin (De

Bruin et al. 2004). Physiological reaction norms studies revealed the extent of phenotypic plasticity and the underlying genetic diversity among genotypes isolated from different locations (Gallagher et al. 1984) but also among genotypes isolated from one location, one time point samples (Brand 1982, Brand et al. 1981, Rynearson and Armbrust 2000, 2004).

The effects of temperature may be confounded with effects of cell size in diatoms. Each diatom cell is encased by two silicate frustules, a smaller hypo- and a larger epitheca, whereby the thecae overlap in the girdle zone (Macdonald 1869). With each mitotic cell division, each of the thecae will form the epitheca of the daughter cells, which results in gradual shrinking of the cell size of the diatom population (Lauterborn 1896, Macdonald 1869). Increased numbers of cell cycles at warmer temperatures therefore will lead to a smaller sized population. But also sudden cell size reductions can occur (Vanormelingen et al. 2007). Size restitution in diatoms can be achieved through sexual reproduction events and has been recorded for a number of diatoms amongst others for *Thalassiosira weissflogii* Grunow (Armbrust and Chisholm 1992) but not for *Asterionella formosa* (Maberly et al. 1994, Mann 1988). Vegetative cell size restitution has been described in *Grammatophora marina* (Lyngbye) Kützing (Sato et al. 2008), but again is unresolved in *Asterionella*. Cell size is directly related to many physiological processes and therefore has effects on metabolic rates (Finkel 2001, Kleiber 1947). Additionally, cell volume and cell surface to volume ratio (SA/V) affect nutrient uptake and within cell diffusion. Whether temperature effects may reduce cell size beyond the effect of increased cell cycles (expressed as change per generation) is not clear.

In the present study, we focussed on *Asterionella formosa* Hassall, a cosmopolitan freshwater pennate diatom that forms stellate colonies of generally four to 8 cells by clonal reproduction. *Asterionella* is an important spring bloom species (Lund 1949) in oligo- and

mesotrophic lakes (see references in (Soudek and Robinson 1983)). Many AFLP based genotypes of *Asterionella* have been shown to co-exist in Lake Maarsseveen (De Bruin et al. 2004), despite the presumed lack of sexual reproduction in *Asterionella* (Mann 1988) and restricted gene flow in the lake. *Asterionella* does not produce spores or cysts nor does it appear particularly desiccation resistant, hence dispersal via air or water birds is not likely to play a large role (K. M. Atkinson 1980, Kristiansen 1996). Additionally, Lake Maarsseveen is rather isolated as it has no direct inflowing connections to other surface waters (Ringelberg 1981).Here we report the results of a reaction norm experiment of seven co-occurring *Asterionella formosa* genotypes across an ecologically relevant temperature range and describe GxE interactions in maximum population growth rates and cell size trait changes per generation. Further, we used the genotype and temperature specific maximum population growth rates to simulate relative proportions of the different genotypes over the course of a year with different temperature scenarios. Our work extends the knowledge of the ecological basis for the maintenance of intraspecific genetic diversity in microorganisms through the potential for sequential selection of different genotypes in variable environments.

Materials and Methods

Isolation

Seven *Asterionella formosa* genotypes were isolated from a single water sample taken with a 5L Uwitec water sampler during an *Asterionella* spring bloom in January 2008 at 5m depth in Lake Maarsseveen. Lake Maarsseveen (52.142828 N, 5.085711 E, The Netherlands) is a dimictic, mesotrophic lake with a maximal depth of 32m and its further general limnology is reported by Ringelberg (1981). Within 4h after sampling, single colonies were isolated and transferred to

modified CHU-10 medium (Stein 1973) enriched with twofold concentrations of PO_4 and $FeCl_3$. As *Asterionella* colonies consist of uniclonal cells only, isolating single colonies is a simple way to achieve uniclonal cultures. Culture establishment from isolated colonies was fairly unbiased with a larger than 95% success rate. The isolates were maintained in uniclonal batch cultures in Sanyo versatile environment test chambers (Sanyo Electrics Co., Ltd., Japan) at 18°C ± 1°C and discontinuous illumination on a 14:10h light:dark cycle at 50µmol quanta s^{-1} m^{-2} provided by cool white fluorescent lamps (Master TL-D 30W/830, Philips, The Netherlands). Light intensity was measured with a Li-Cor LI-189 quantum meter (LI-COR, inc., Lincoln, USA). All genotype cultures had slight bacterial contaminations.

Genetic analysis

To assess the genetic diversity among *Asterionella formosa* genotypes they were fingerprinted by AFLP markers. AFLPs are sensitive and reproducible markers well suited to reveal genotypic and phylogenetic relationships (Rademaker et al. 2000) and are able to provide useful information about population genetic structure where other markers lack sufficient resolution, for instance to characterise clonal structure of a single algal bloom sample (John et al. 2004). *Asterionella* DNA was extracted with a Qiagen DNeasy Plant Mini Kit (Qiagen BV., The Netherlands) with a modification of the lysis step. Fifty mL of dense *Asterionella* cultures were centrifuged at 740 x g for 10 min. The supernatant was discarded and the remaining pellet was transferred into a fresh tube with 0,5g of 0.1mm zirconium beads and 300µl lysis buffer which consisted of 10 mM Tris 1M, 1 mM EDTA pH8 and 0.5% SDS. Then the sample was bead-beaten three times with a mini bead beater (Biospec Products, Bartlesville, USA) at 840 x g for 30 sec per round and cooled on ice between each round. After adding 100µg mL^{-1} Proteinase K and short vortexing, the sample was incubated at 60°C for one hour and then processed further

following the Qiagen DNeasy Plant Mini Kit protocol from the first precipitation step onwards. The AFLP analysis was performed by Keygene® (Wageningen, The Netherlands) using the same four EcoRI/MseI AFLP primer combinations as reported in De Bruin et al. (2004), a) Eco+GA & Mse+AT, b) Eco+GA & Mse+CC, c) Eco+GA & Mse+CG, d) Eco+GC & Mse+AC.

Experimental design

The experiment was conducted using a full factorial design with seven *Asterionella* genotypes (S24, S26, S37, S38, S43, S49 and S53) and five different temperatures (1°C, 6°C, 11°C, 16°C or 21°C ± 0.5°C) in six replicates per treatment combination, resulting in 35 experimental combinations and 210 experimental units. The temperature levels represent the full range of temperatures experienced by the *Asterionella* population in Lake Maarsseveen over the course of a year (Gsell et al. 2012). All cultures were placed in temperature controlled water-baths and light was set to discontinuous illumination at 160±10 µmol quanta m^{-2} s^{-1} provided by cool white fluorescent lamps on a 14:10 light: dark cycle. Measurements of the photosynthesis vs irradiance curves on a PhytoPam (Heinz Walz GmbH, Germany) showed that this irradiance level was saturating but not inhibiting for all seven genotypes. All genotype cultures were acclimatised stepwise to their experimental temperature so that each genotype culture spent between 12 and 42 generations in semi-continuous batch culture at their experimental temperature prior to the start of the experiment (i.e. 2 weeks for the 21°C cultures to 13 weeks for the 6°C cultures). All experimental treatments were started on the same day. Six replicates of each clonal subculture were inoculated at about 10 000 cells mL^{-1} in 60mL CHU-10 medium. This inoculum size was small enough to ensure that the culture medium could support several generations of population growth before either light or nutrient availability could become limiting (i.e. before the culture would enter stationary growth phase). The cultures were shaken twice per day and their positions

randomized within their water bath once each day. Samples were taken on days 0, 1 and 2, after that on every other day until day 12 for temperature treatments 11°C to 21°C and at days 0, 1, 3, and every third day until day 21 for temperature treatments 1°C and 6°C, all at the same moment of the light cycle. The samples were fixed with a Glutaraldehyde-Formaldehyde fixative and stored cool and in the dark.

The number of *Asterionella* cells mL^{-1} was calculated from optical density measurements at 720nm in 5cm cuvettes on a Lambda 800 UV/VIS spectrometer (Perkin Elmer Instruments, United Kingdom) whereby the specific light extinction to number of cells conversion factor was determined for each genotype-temperature combination separately. Maximum population growth rates were calculated by fitting a least squares linear regression to the linear part of each time course of natural log transformed algal densities. Cell length and width (in µm) were measured for 30 cells each of all six replicates per genotype–temperature combination using an inverted microscope (DMI 4000B, Leica Microsystems CMS GmbH, Wetzlar, Germany) and the image analysis program Cell-D (Olympus Soft Imaging System GmbH, Münster, Germany). Care was taken to measure no cells in the process of division, as this would inflate the width measurements. Surface-area to volume ratio (SA/V) and biovolume were calculated from length and width measurements whereby the *Asterionella* cell was assumed to have the form of an elongated cuboid. The change in cell length, width, biovolume and SA/V per generation was calculated by subtracting the averages of day 0 samples from that of day 9 (for 1°C treatments) and day 10 (for 11 and 21°C treatments) samples and by dividing that difference by the temperature and genotype specific generation time for all six replicates of all genotypes at temperature treatments 1°C, 11°C and 21°C. Hence, negative values mean that the cell trait has decreased over time; positive values mean that the cell trait has increased over time. Number of

cells per colony was counted for 30 colonies for all genotypes at temperature treatments 1°C, 11°C and 21°C in four out of six replicates on day 12 samples.

Statistical analysis

For the AFLP analysis each locus was treated as a separate character and scored for presence or absence, yielding a binary data matrix. This matrix was used to estimate genetic similarity of all possible pair-wise combinations of the tested genotypes using Jaccard's similarity coefficient (Jaccard 1901). Jaccard's similarity ignores shared absences and the coefficient ranges from zero (no similarity) to one (complete similarity) (Bonin et al. 2007). Cluster analysis using the UPGMA procedure (unweighted pair-group method using arithmetic means) was performed to generate a dendrogram visualising the similarity of the tested genotypes. A cophenetic matrix was compared to the Jaccard's similarity matrix and the cophenetic correlation coefficient was calculated to evaluate to what extent the similarity matrix supported the dendrogram (Rohlf and Sokal 1981).

We used a factorial ANOVA to test for main and interaction effects on growth rates, cell length, width, biovolume and SA/V and number of cells per colony with temperature as a fixed factor and genotype as random factor. All data were checked for normality and heteroscedasticity. Dependent variables were square root transformed if needed to meet the assumptions of ANOVA. Since transformations did not remove non-normality in the population growth rate variable, the raw data were used for the analysis with a more conservative significance level of ≤ 0.01 (Fowler-Walker and Connell 2002). Post Hoc Tukey's HSD tests were used to show the temperature effect in more detail. Correlations of cell length and width, as well as of genetic and phenotypic similarity were calculated using the Pearson r coefficient. All statistical analyses were run in Statistica 8.0 (Stat Soft Inc. 2008, Tulsa, USA), or in the

multivariate analysis program NTSYSpc 2.11C (Applied Biostatistics Inc., USA). Bray Curtis similarity indices for phenotypic similarity on population growth rates were calculated with the program EstimateS (Colwell 2009). In order to measure the magnitude of the genotype effect on the population growth response, Cohen's D effect sizes were calculated per temperature as each possible pair-wise mean trait difference divided by their pooled standard deviation. The five Cohen's D values for each pair-wise genotype comparison where then averaged to yield an overall Cohen's D per pair-wise comparison. The cut off values for classification of effect sizes were adopted from Ferguson *et al*. (2009) thus effects sizes of ≥ 0.41 fell in the category "practically significant", effects sizes of ≥ 1.15 were considered "moderate" and effect sizes of ≥ 2.7 were "strong".

Simulation model

A straightforward simulation model of the relative population proportion of each *Asterionella* genotype was constructed. The model was based on the average maximum growth rates of each genotype at each temperature and run with actual seasonal surface water temperature patterns of Lake Maarsseveen in order to simulate whether different genotypes may be favoured sequentially as the environmental conditions change with the seasons. The simulated population was started with equal proportions of each of the seven genotypes. The population growth rate of each genotype at any given temperature was based on linear interpolation between the experimentally measured population growth rates at the two closest experimental temperatures. Because records of water temperature in Lake Maarsseveen were incomplete, an existing model was used to predict the surface water temperatures from air temperature records (Mooij et al. 2008). These predictions of yearly temperature patterns were then used to force the population growth rate responses for the duration of a calendar year resulting in relative densities of each

genotype. In order to categorise (as described in (De Senerpont Domis et al. 2003)) the yearly temperature patterns, and thereby the resulting patterns of genotype frequency, the Bray-Curtis similarity among years was calculated in the program EstimateS (Colwell 2009)). These similarities were used to conduct a PCoA (Gower 1966) in the program NTSys (Exeter software, USA). To identify meaningful classification parameters Kendall τ correlations (Kendall 1948) were calculated between the loadings of the first three PCoA axes and average temperature of hibernal, prevernal, vernal, estival, serotinal and autumnal periods.

Results

Genotyping

The four AFLP primer combinations selected yielded an average of 33.2 ± 2.2 marker bands per primer pair and a total of 133 marker bands of which 30.8% were polymorphic. Each uniclonal culture showed a unique banding pattern and therefore represented a unique genotype. The UPGMA dendrogram (Fig. 1) showed two clusters of three and four genotypes and was a fair representation of the Jaccard's similarity matrix as the cophenetic correlation coefficient was r = 0.85 (approximate Mantel t-test $p = 0.99$).

Population growth rates

The average population growth rate per day of the seven *Asterionella* genotypes ranged from 0.14 ± 0.007 (S.D.) at 1°C to 0.65 ± 0.021 (S.D.) at 21°C (Fig. 2a). This equalled a doubling time of 6.69 to 0.98 days. Inoculation densities of all treatments showed some spread ranging from 8000 to 23 000 cells mL^{-1}; however these differences in density showed no consistent effect on population growth rates (data not shown). Unfortunately population growth rates could not be transformed to conform to normality; therefore we employed a more conservative *p*-value of 0.01. Still, the ANOVA detected a highly significant temperature effect ($F_{4, 24}= 38.81$, $p < 0.001$)

as well as a significant genotype by temperature (GxE) interaction ($F_{24, 175} = 57.89$, $p < 0.001$) but no direct genotype effect on growth rate, (for all ANOVA results see Table 1). Post Hoc Tukey's HSD tests showed that, on average, all genotypes grew significantly faster with increasing temperatures (21ºC > 16ºC > 11ºC > 6ºC > 1ºC, $p < 0.0001$). However, the ranking order of genotypes changed for each temperature. Almost all genotypes except the combination of S43 and S49 expressed a unique phenotypic response curve as indicated by pair wise comparisons using Post Hoc Tukey's HSD test.

Per generation changes in cell size traits

The average genotype specific changes in cell length per generation (Fig. 2b) ranged from +0.67 µm ± 0.16 (S.D.) at 1°C to -2.57 µm ± 0.15 (S.D.) at 21°C. The ANOVA on changes in average cell length per generation showed a significant GxE interaction ($F_{24, 175} = 18.71$, $p < 0.001$), but no direct genotype or temperature effect. Average cell lengths at day 9 and 10 ranged from 22.6 to 52.8 µm (see Fig. S2a in supplementary materials). Mean cell length at day 0 correlated negatively with the mean change in length per generation, but the fraction of explained variance of the correlation was rather low (pearson's r = -3.72, $p < 0.05$, $R_{adj}^2 = 0.19$). Each genotype differed in mean cell length and longer cells generally were less wide (pearson's r = -0.43, $p < 0.05$).

The changes in cell width per generation (Fig. 2c) ranged from +0.393µm ± 0.023 (S.D.) at 1°C to -0.013 µm ± 0.004 (S.D.) at 11°C. The ANOVA showed a significant temperature ($F_{2, 12} = 138.06$, $p < 0.001$) effect and a significant GxE interaction ($F_{12, 105} = 3.62$, $p < 0.001$) but no direct genotype effect. Generally, cells increased their width per generation significantly at the lowest temperature as shown by significant Post Hoc Tukey's HSD tests for 1°C > 11°C, $p < 0.001$ and for 1°C > 21°C, $p < 0.001$. Per generation change in width was not significantly

different among 11ºC and 21ºC treatments. Cell widths on day 9 and 10 ranged from 2.28 to 3.36 µm (Fig. S2b).

Changes in cell volume per generation (Fig. 2d) ranged from +92.95 μm^3 ± 4.554 (S.D.) at 1°C to -12.003 μm^3 ± 2.304 (S.D.) at 11°C. ANOVA results showed significant temperature effects ($F_{2, 12} = 53.69$, $p < 0.001$) and a significant GxE interaction ($F_{12, 105} = 11.44$, $p < 0.001$) but no direct genotype effect. Generally cell volumes increased most per generation at the lowest temperature as shown by significant Post Hoc Tukey's HSD tests for 1°C > 11°C, $p < 0.001$ and for 1°C > 21°C, $p < 0.001$. Per generation changes in cell volume were not significantly different among 11ºC and 21ºC treatments. Cell volume at day 9 and 10 ranged from 206.3 to 510.4 μm^3 (Fig. S2c).

Changes in SA/V per generation (Fig. 2e) ranged from +0.009 ± 0.003 (S.D.) at 11°C to -0.28 ± 0.019 (S.D.) at 1°C. The ANOVA detected a significant temperature ($F_{2, 12} = 46.31$, $p < 0.001$) and a significant GxE interaction effect ($F_{12, 105} = 10.45$, $p < 0.001$) but no direct genotype effect. The general response of cell SA/V change per generation showed the strongest reduction in SA/V at 1°C as cells got wider and therefore contained more volume per surface area as shown by the significant Post Hoc Tukey's HSD test 1°C > 11°C, $p < 0.001$ and 1°C > 21°C, $p < 0.001$. Again, per generation changes in cell SA/V were not significantly different among 11ºC and 21ºC treatments. The SA/V at day 9 and 10 ranged from 1.29 to 1.84 (Fig. S2d).

Cell number per colony

Cell numbers per colony at day 12 ranged from 1 to 16 cells with a median of 4 cells per colony at 1°C and 8 cells per colony from 11°C to 21°C (Fig. 2f). The cell per colony data was borderline heteroscedastic (Bartlett Chi^2=55.40, p=0.012). Therefore again a more conservative *p* -value of 0.01 was employed. Still the data suggested a temperature ($F_{1, 12} = 36.74$, p=< 0.001)

and a GxE ($F_{12, 63}$ = 35.56, p=< 0.001) effect. Cell numbers per colony increased with temperature as shown by significant Post Hoc Tukey's HSD tests for 21°C > 11°C > 1°C, p = 0.0001.

Phenotypic similarity and effect sizes

Pearson's r correlation between genetic similarity (derived from Jaccard's similarity on AFLP data) and phenotypic similarity (derived from Bray Curtis similarity on growth rate as fitness measure) showed that higher genetic similarity correlated positively with higher phenotypic similarity (Pearson's r = 0.54, R^2_{adj} = 0.29, p = 0.011, Fig. 3). Cohen's D effect sizes for average differences in growth rates showed moderate (cut off value ≥ 1.15) to strong (cut off value ≥ 2.7) effect sizes for half of the genotype pair comparisons (Table 2).

Simulation model

The simulation model was started with equal proportions of all seven genotypes and run with surface temperature patterns of different years in Lake Maarsseveen. In order to identify meaningful classification parameters for the different years Kendall τ correlations were calculated between the loadings of the first three axes of the PCoA and average temperature of hibernal, prevernal, vernal, estival, serotinal and autumnal periods, (see Fig. S1 in supplementary materials for a three dimensional representation of the ordination). Axis 1 explained 62.3% of the variation and correlated best with average hibernal temperature (Kendall τ = 0.69, Z = 6.89, p < 0.0001), axis 2 explained 22.4% of the variation and correlated best with average estival temperature (Kendall τ = 0.49, Z = 4.97, p < 0.0001) and axis 3 explained 15.2% of the variation and correlated best with average vernal temperature (Kendall τ = 0.43, Z = 4.36, p < 0.0001). Based on the hibernal axis, all years were roughly classified into colder or warmer winter years. Depending on the annual temperature pattern underlying the simulation, the model resulted in a

successive numerical dominance of two to several genotypes (Fig. 4). Years with colder winters (such as years 1970, 1987 and 1996) were more likely to show a prolonged coexistence of all seven genotypes over the first 50 to 100 days, although the relative abundance of each genotype still varied considerably. Years with warmer winters (such as 2003) were more likely to show early and lasting numerical dominance of only one or two *Asterionella* genotypes (Fig. 4).

Discussion

Thermal reaction norms in fitness and rates of morphological change

GxE experiments usually examine the reaction norms of genotypes from a wide origin to maximize the effect of either geographic or temporal distance (Lakeman et al. 2009) while the effect of environmental variation on the relative fitness of genotypes co-occurring at a single time point and location has been tackled less often (but see (Brand 1982, Brand et al. 1981, Rynearson and Armbrust 2000, 2004). Our reaction norms results reveal considerable physiological diversity in seven co-occurring genotypes originating from an isolated population of the predominantly clonally reproducing diatom *Asterionella formosa*. Significant GxE interactions with large η^2 values indicate that the level of the growth response is context dependent in such a way that it is not possible to predict the ranking order of the response from one environment to the next. Environmental variation such as temperature change may therefore provide an example of fluctuating selection sequentially favouring different genotypes (Sebens and Thorne 1985). Other studies on reaction norms of genotypes originating from a single location and point in time samples have also shown GxE interactions: Brand (1981) found variation in thermal reaction norms of growth rate in five co-occurring genotypes of *Thalassiosira pseudonana*. However he attributed this variation to these genotypes belonging to different ecotypes as they were sampled from a warm core eddy overlying slope water, a region

where neritic and oceanic populations were mixed (Brand et al. 1981). Rynearson and Armbrust (Rynearson and Armbrust 2000, 2004) tested several *Ditylum brightwellii* genotypes originating from three hydrologically connected populations and found distinct reaction norms and GxE interactions across a light gradient. Our results agree with the findings of these studies and support that in heterogeneous environments the relative success of any given genotype may constantly shift. Population genetic diversity may be maintained if the environmental variation recurs in cycles (such as yearly seasonal temperature changes) as all genotypes experience favourable and unfavourable periods and therefore are able to persist (Gallagher 1980, Rynearson and Armbrust 2005).

In predominantly (or fully – see (Mann 1988)) asexually reproducing species such as *Asterionella*, population growth rate can be regarded as a measure of fitness (Wood 2005) as it is the net outcome of all factors governing survival and reproductive success. The population growth rates for *Asterionella* in our experiment ranged well within *Asterionella* population growth rates published elsewhere (Bruning 1991, Butterwick et al. 2005, Lund 1949, Rhee and Gotham 1981, Tilman and Kilham 1976, Van Donk and Kilham 1990). All genotypes were able to cope with the full range of experimental temperatures. Although no significant direct effect of the genotype on fitness was observed, each genotype reacted differently to the different temperature environments. There was no genotype that "out-competed" all others consistently across all temperatures as the fitness ranking order of genotypes changed with temperature environment. Genetically more similar genotypes displayed phenotypically more similar reaction norms as shown by a correlation between genetic similarity (based on Jaccard's similarity of AFLP data) and phenotypic similarity (based on Bray Curtis similarity of population growth rate reaction norms). This suggests a genetic basis for phenotypic plasticity in growth rates. A similar

finding was presented by Jasienski *et al.* (1997) in annual plants. Additionally, in terms of growth rate effect sizes, the genotypes differed moderately to strongly, suggesting that these differences in reaction norms may be ecologically relevant.

Changes in cell size per generation

Algae cell size is directly related to many physiological processes and therefore has effects on metabolic rate (Finkel 2001, Kleiber 1947), photosynthetic capacity (Finkel 2001, Raven and Kubler 2002) and respiratory rate (Tang and Peters 1995). Furthermore, in many diatom species sexual reproduction is initiated when the cells reach a minimum size (Nipkow 1927). Additionally, cell size has implications for the risk of predation as smaller cells are more easily ingested (Smetacek et al. 2004) whereas larger diatoms seem to be preferred by chytrid parasites (Ibelings et al., 2004). Larger cells are more prone to sinking (Kiorboe 1993) although form resistance of colonies improves the buoyancy of large diatoms (Jaworski et al. 1988). Actively metabolizing large cells may also have other means to improve their buoyancy (Waite et al. 1997). Cell size, more specifically cell volume and radius are thought to have implications for nutrient uptake rate (Aksnes and Egge 1991, Irwin et al. 2006, Kiorboe 1993, Sunda and Huntsman 1997), as cells with a larger radius are more likely to encounter a diffusion limit. A larger surface area also means more space for trans-membrane nutrient transporters and cells with higher SA/V can extract nutrients more efficiently from the environment (Finenko and Krupatkina-Akinina 1974), therefore non-spherical cells with a larger SA/V may in fact profit from being larger. Size and shape of cells are therefore also the main constituents of functional classifications of phytoplankton (Kruk et al. 2010, Reynolds et al. 2002).

We measured the phenotypic change in cell size per generation across the full range of temperatures encountered by *Asterionella* over the seasons to evaluate the relative effect of

temperature and genotype on the rate of change. The most noticeable change was a higher rate of increase in average cell width at the lowest temperature. At low temperatures and slow population growth, cells may accumulate more nutrients and photosynthates before division. The accumulated storage products may thus be accommodated by expanding the width of the girdle zone (Macdonald 1869, Round et al. 1990). Rates of change in cell length per generation did not show clear temperature dependence but genotype specific responses differed widely at different temperatures. Generally actual cell lengths were shorter at the higher temperature treatments (see supplementary materials), partly a result of the number of cell cycles but potentially also of an increased chance of abrupt cell size reduction due to faulty cell divisions. Different rates of change in cell width also affect the rates of change in cell volume and SA/V ratio, and therefore have consequences for nutrient uptake capacities (Winder et al. 2009). At the lowest temperature the biovolume increased markedly while the SA/V decreased as the cells became ever so slightly more spherical. Smaller size compensates for the decreased ratio of supply versus consumption of potentially limiting resources like phosphorus or carbon dioxide with an increase in temperature (D. Atkinson et al. 2003).

The number of cells per colony reportedly depends on several environmental factors. While cell numbers per colony decrease with phosphate limitation (Tilman et al. 1976), they increase with light intensity (Wagner 2008), warmer temperature (Hayakawa et al. 1994) and silicate limitation (Tilman et al. 1976). Observations of seasonal changes in cell number per colony in natural populations of *Asterionella formosa* support the hypothesised role of light and temperature conditions. Lund (Lund et al. 1963) and Hayakawa (Hayakawa et al. 1994) reported both a shift from four- or six-celled colonies in winter and early spring to 8- or more-celled colonies in summer. *Asterionella* colonies are formed through asexual reproduction of cells,

whereby the cells remain connected by mucilage pads that are excreted at the apex of the cells. The excretion of the mucilage seems to be a by-product of photosynthesis and therefore might be light dependent (Hayakawa et al. 1994). However, enzyme catalysed processes occurring downstream of light absorption such as photophosphorylation, and the Calvin cycle are temperature dependent (Raven and Geider 1988). Hence temperature may also play a role in the shift of mean number of cells per colony observed in the experiment from about four cells at 1 °C to about 8 cells at 21 °C. Again, the genotype effect depended on the temperature level which means that genotypes reacted not uniformly to the temperature change. Colony size has direct implications for sinking rates through changes in form resistance and for the risk of grazing by zooplankton (Wagner 2008). The rate at which genotypes make the shift from the more vulnerable four-celled constellation to the better protected 8-celled constellation may have ecological relevance.

Simulation

The simulation model showed that the GxE interactions in the population growth rates produced different patterns of relative proportions of each genotype in the population, depending on the underlying temperature pattern. Generally, the simulation resulted in a succession of two to several genotypes in time. Winter temperatures were the most prominent factor differentiating among years while summer temperatures seemed to be less variable. Further, winter temperature patterns seemed to be particularly important in determining genotypic succession patterns. Cold winters prolonged coexistence of genotypes with a succession of several genotypes becoming numerically dominant while the other genotypes still persisted in the population. Warmer winters seemed to lead to an early and prolonged dominance of one or two genotypes only.

The seven genotypes, tested in the experiment and simulation, are only a small subsample of the entire *Asterionella* population of genotypes present in Lake Maarsseveen. AFLP fingerprinting surveys of the *Asterionella* population in the same lake showed high genetic diversity as none of the 41 sampled genotypes was sampled twice (De Bruin et al. 2004). Extrapolating from the experimental and simulation results, each extant genotype can be expected to show its own interaction pattern with an environmental modulator of genotype fitness such as temperature, resulting in more intricate succession pattern of genotypes. Temperature has direct effects on cell physiology through impacting the properties of a range of catalysts and membrane lipids (Raven and Geider 1988). However, surface water temperature is only one of many possible environmental modulators of genotype fitness. Light intensity (Rynearson and Armbrust 2000) and genotype specific parasitism (De Bruin et al. 2004) can also act as modulators of fitness and therefore impact the strength and direction of clonal selection. Additionally, temperature can also interact with other modulators such as parasitism to alter host genotypic fitness, for instance when the level of genotypic resistance to parasitism depends on the level of the temperature environment (Mitchell et al. 2005).

The combination of experimental data on GxE interactions in concurrent *Asterionella* genotypes across an ecologically relevant range of temperatures and of a simple simulation approach, allow showing how relative success of each genotype constantly shifts in a temporally variable environment, However, the outcome and strength of the clonal selection is influenced by the timing and duration of thermal patterns such as colder or warmer winters. If such fluctuating selection is operating in cyclical environments such as recurring seasonal temperature cycles, this may provide an example of balancing selection in the sense of Sebens and Thorne (1985):

even if the balance is not exact, it may promote prolonged coexistence. Thus clonal diversity may be maintained by physical variation in the system.

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Figure legends

Figure 1

AFLP genotyping results: A UPGMA based dendrogram representation of the Jaccard's similarity matrix of the seven tested genotypes

Figure 2

Reaction norms of seven *Asterionella formosa* genotypes across a temperature gradient: panel a) population growth rate (d^{-1}); panel b) change in cell length per generation (µm); panel c) change in cell width per generation (µm); panel d) change in cell biovolume per generation (μm^3); panel e) change in surface to biovolume ratio per generation of cells; and panel f) number of cells per colony. Error bars indicate standard error of the mean and may be hidden under the symbols.

Figure 3

Phenotypic similarity and genotypic similarity correlate positively

Figure 4

Simulation of changes in relative proportion of *Asterionella* genotypes over a year based on genotype and temperature specific population growth rates and forced by surface water temperature patterns of different years in Lake Maarsseveen. Panels 1970, 1987 and 1996 show years with cold winters, panel 2003 shows a year with a warm winter. Left panels show the simulation output, right panels the surface water temperature pattern for the same year.

Table 1: Results of factorial ANOVA analysis with temperature (E) as fixed and genotype (G) as random factors. Significance level (*) for all dependent variables is p = 0.05, but for non normal distributed or borderline heteroscedastic dependents (population growth rate and cells / colony) a more conservative significance level of p = 0.01 is implemented.

variable	effect	p	df	F	Part. eta^2
Growth rate	E	< 0.001*	4	68.95	0.92
Growth rate	G	0.48	6	1.00	0.20
Growth rate	GxE	< 0.001*	24	47.68	0.87
Cell length	E	0.41	4	0.96	0.14
Cell length	G	0.81	6	0.48	0.19
Cell length	GxE	< 0.001*	24	18.72	0.68
Cell width	E	< 0.001*	4	138.1	0.96
Cell width	G	0.13	6	2.08	0.51
Cell width	GxE	< 0.001*	24	3.62	0.29
Cell biovolume	E	< 0.001*	4	53.69	0.89
Cell biovolume	G	0.76	6	0.55	0.21
Cell biovolume	GxE	< 0.001*	24	11.44	0.57
SA/V	E	< 0.001*	4	46.32	0.89
SA/V	G	0.29	6	1.38	0.41
SA/V	GxE	< 0.001*	24	10.45	0.54
Cells /colony	E	< 0.001*	2	36.74	0.89
Cells /colony	G	0.07	6	2.67	0.57
Cells /colony	GxE	< 0.001*	12	35.56	0.87

Table 2: Cohen's D effect sizes for pair-wise comparisons of genotype specific population growth rate averaged over all temperatures. Grey shades code for increasing effects from low, moderate to strong effects with increasing intensity.

	S24	S26	S37	S38	S43	S49	S53
S24		-2.064	-1.036	-0.676	0.604	-1.382	2.002
S26			0.768	1.497	1.191	0.778	3.182
S37				0.284	-0.491	-0.481	2.302
S38					-0.071	-0.384	2.486
S43						0.457	2.867
S49							2.802
S53							

Figures:

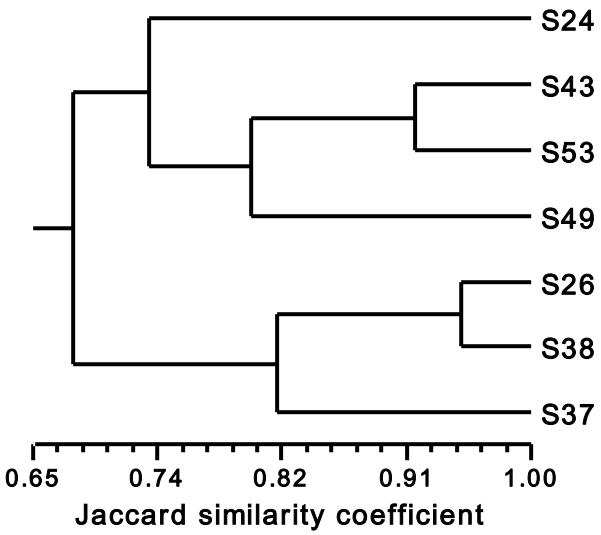

Figure 1

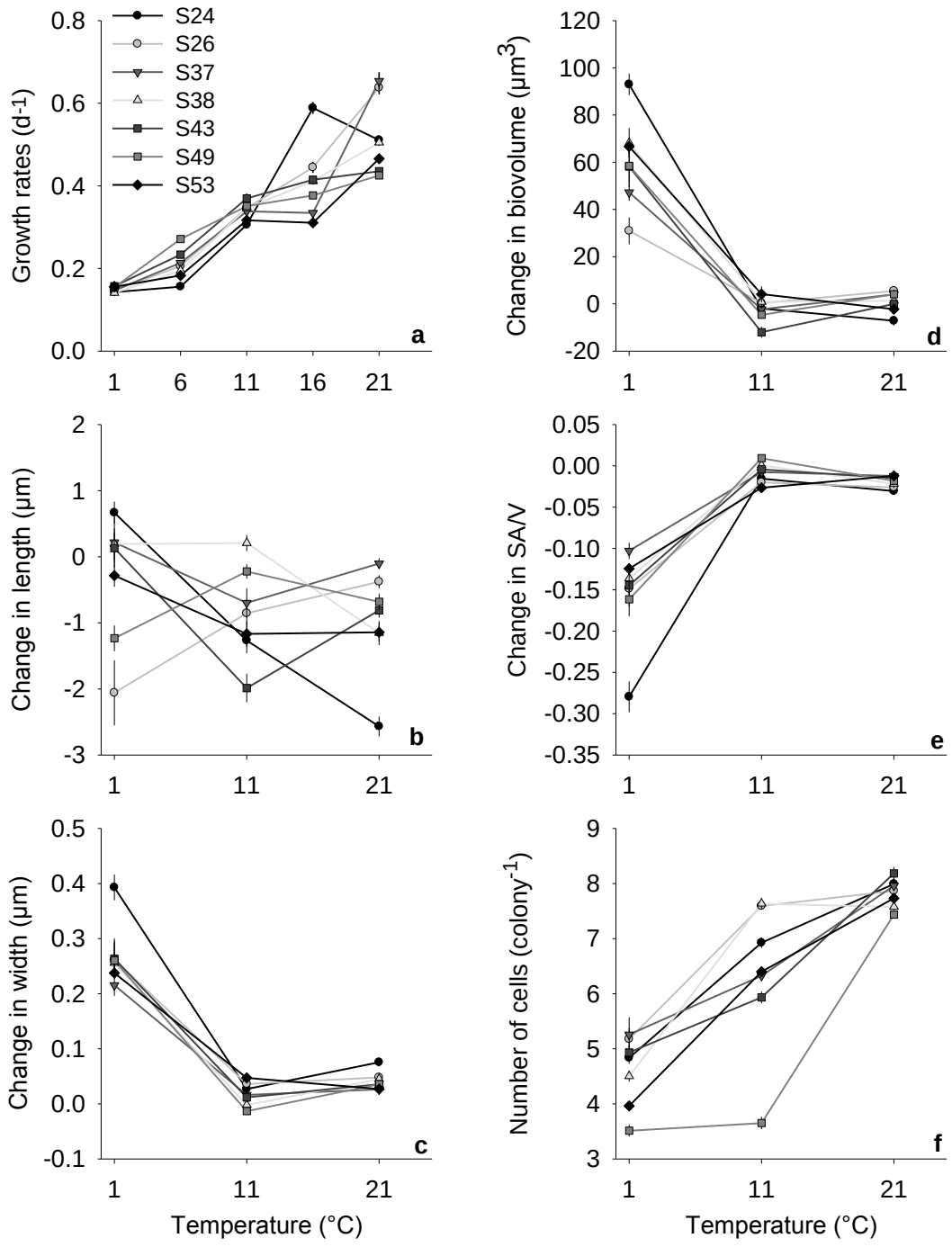
S24
S26
S37
S38
S43
S49
S53
Growth rates (d-1)
Change in biovolume (µm3)
Change in length (µm)
Change in SA/V
Change in width (µm)
Number of cells (colony-1)
Temperature (°C)
Temperature (°C)
a
b
c
d
e
f

Figure 2

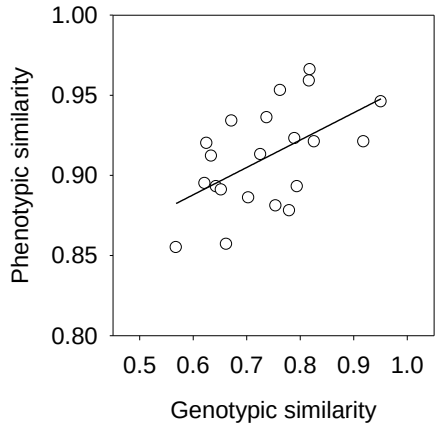
1.00
0.95
0.90
0.85
0.80
Phenotypic similarity
0.5
0.6
0.7
0.8
0.9
1.0
Genotypic similarity

Figure 3

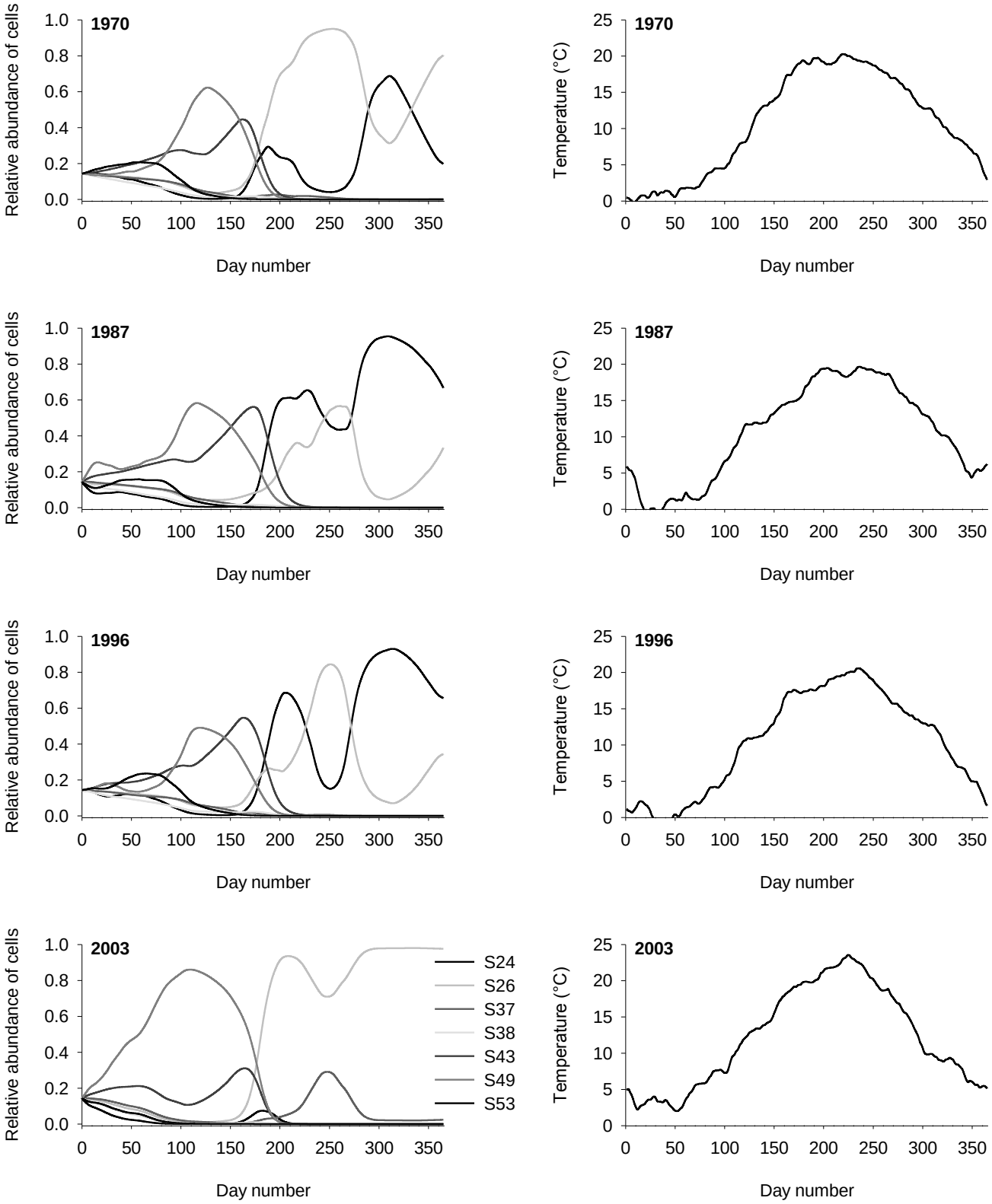
1970
1987
1996
2003
Relative abundance of cells
Temperature (°C)
Day number
S24
S26
S37
S38
S43
S49
S53

Figure 4

Supplementary materials

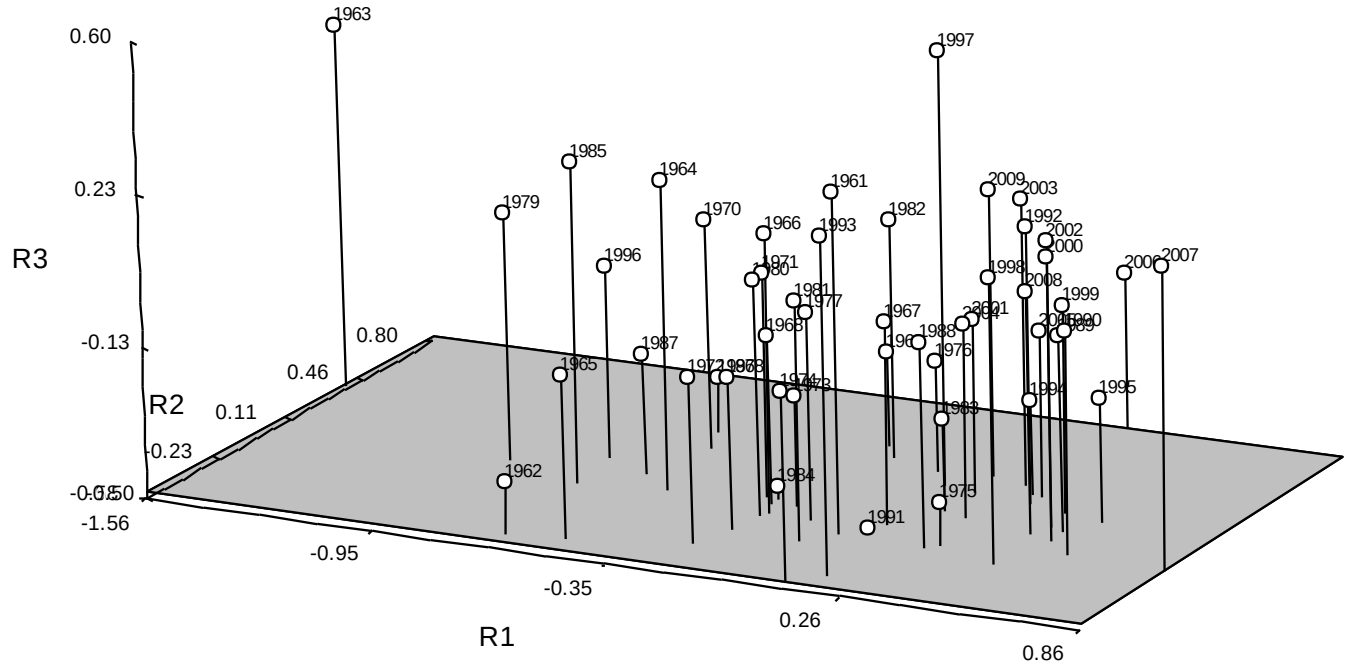

Figure S1: Classification of years based on a PCoA of yearly water temperature patterns. Axis 1 explained 62.3% of the variation and correlated best with average hibernal temperature, axis 2 explained 22.4% of the variation and correlated best with average estival temperature and axis 3 explained 15.2% of the variation and correlated best with average vernal temperature. Based on the hibernal axis, all years were roughly classified into colder or warmer winter years.

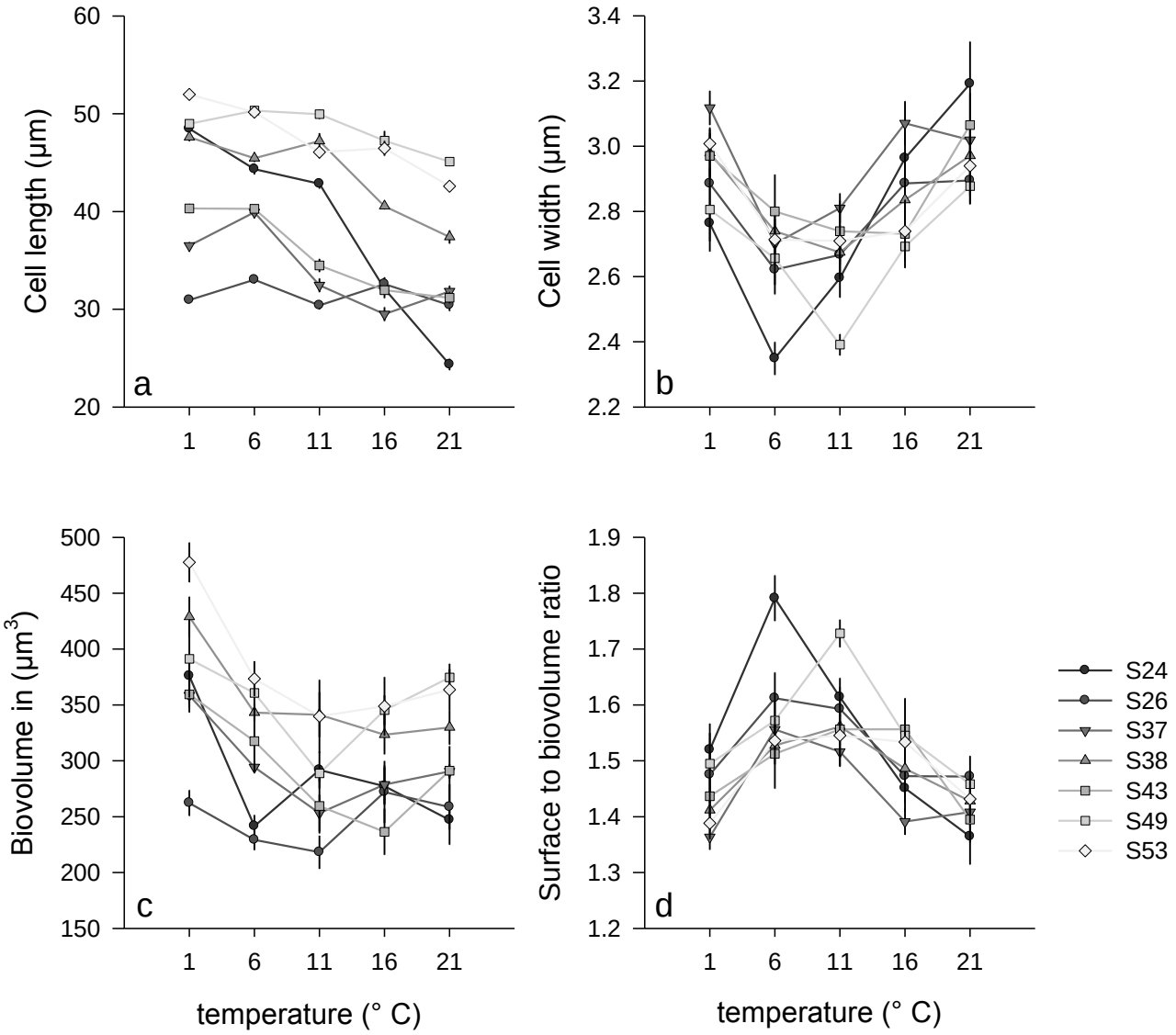

Figure S2: Actual size measurements per genotype and temperature as measured on day 9 (1 and 6°C treatments) and day 10 (11-21°C treatments) for panel a) cell length in µm, panel b) cell width in µm, panel c) cell biovolume in μm^3 and panel d) cell surface to volume ratio.