ORIGINAL ARTICLE

Single-cell mass spectrometry reveals the importance of genetic diversity and plasticity for phenotypic variation in nitrogen-limited *Chlamydomonas*

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Phenotypic variation is vital for microbial populations to survive environmental perturbations. Both genetic and non-genetic factors contribute to an organism's phenotypic variation and therefore its fitness. To investigate the correlation between genetic diversity and phenotypic variation, we applied our recently developed mass spectrometry method that allows for the simultaneous measurement of more than 25 different lipids and pigments with high throughput in the unicellular microalga *Chlamydomonas reinhardtii*. We monitored the impact of nitrogen limitation on a genetically diverse wild-type strain CC-1690 and two isoclonal isolates from CC-1690 named ANC3 and ANC5. Measuring molecular composition of thousands of single cells at different time points of the experiment allowed us to capture a dynamic picture of the phenotypic composition and adaptation of the populations over time. Although the genetically diverse population maintained phenotypic variation over the whole time course of the experiment, the isoclonal cultures showed higher synchronicity in their phenotypic response. Furthermore, the genetically diverse population showed equal or greater phenotypic variation over the whole time range in multidimensional trait space compared with isoclonal populations. However, along individual trait axes non-genetic variance was higher in isoclonal populations.

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Introduction

The ability of organisms to express phenotypic variation is a basic feature of life. The expression of phenotypic variation among individuals within a population can increase the chances that some individuals in a population will express adaptive phenotypes in a new or changed environment (Forsman and Wennersten, 2016), and therefore improve the fitness of the species, and increase the chances of its survival (reviewed in Schlichting and Pigliucci, 1998). Phenotypic variation of organisms can result from genomic differences among individuals, but may also occur in isogenic populations due to phenotypic plasticity, epigenetic

effects and bet-hedging, among others (Simons, 2011; Wennersten and Forsman, 2012; Ackermann, 2015). Understanding the relative contributions of genetic and non-genetic sources of phenotypic variation is particularly important in studies of evolution and eco-evolutionary feedbacks for numerous reasons (Dewitt *et al.*, 1998; Hairston *et al.*, 2005; Pigliucci, 2005; Ghalambor *et al.*, 2007; Collins and Gardner, 2009; Auld *et al.*, 2010; Simons, 2011; Yamamichi *et al.*, 2011; Hendry, 2016). First, plasticity and other non-genetic sources of variation may be more limited in the extent of phenotypic variation that they can generate than genetic sources of variation (Dewitt *et al.*, 1998; Gienapp *et al.*, 2008; Auld *et al.*, 2010). Second, genetic and non-genetic phenotypic variation respond to environmental change at different rates, with the expectation that non-genetic sources of variation tend to respond faster (Yamamichi *et al.*, 2011). Last, owing to the first two points above, the relative contributions of each may differ depending on the rate and strength of environmental change observed (Yamamichi *et al.*, 2011; Hendry, 2016). Furthermore, to predict evolutionary responses of populations to future

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environments, an estimate of the genetic basis of phenotypic variance among individuals is required (Schlichting and Pigliucci, 1998). In this paper, we provide the first comparison of the extent to which genetic and non-genetic mechanisms are capable of producing phenotypic variation in a well-studied alga (*Chlamydomonas reinhardtii*) under a well-studied stress (nitrogen limitation) at the level of the single cell.

Although there is a wealth of recently developed methods for extracting single-cell information from genomes (Lu *et al.*, 2012; Zong *et al.*, 2012), transcription (Klein *et al.*, 2015; Macosko *et al.*, 2015) and protein expression (Spitzer and Nolan, 2016), methods for the multiparametric analysis of metabolites in single cells are scarce (Zenobi, 2013). What is more, the great majority of these techniques were developed for mammalian cells. Some methods with single-cell sensitivity have successfully been transferred to microbes, including genome analysis (Rinke *et al.*, 2013; Kashtan *et al.*, 2014; Spencer *et al.*, 2016) and the characterization of phenotypes using fluorescence (Breker *et al.*, 2013; Fontana *et al.*, 2014), Raman microscopy (Schuster *et al.*, 2000) or elemental analysis of isotopes using secondary ion mass spectrometry (Schreiber *et al.*, 2016). However, in general, applying single-cell methods to microbes is challenging due to the great diversity of cell sizes, morphologies and structural properties of cell walls. Therefore, the study of phenotypic variation in microbial systems and its underlying mechanisms is still compromised by technical limitations in microbial systems (Altschuler and Wu, 2010; Ackermann, 2015).

We recently developed a method for the label-free analysis of small molecules in single cells of the microalga *Chlamydomonas reinhardtii*,

which combines matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) with high-throughput microarray sample-preparation technology. This technique, here called single-cell mass spectrometry (SC-MS), showed the reproducible relative quantitative measurements of more than 15 lipids and pigments in thousands of single cells of a unicellular organism for the very first time (Krüger *et al.*, 2015). *Chlamydomonas reinhardtii* is a popular model organism in laboratory evolution experiments owing to the tools developed for transformation and sequencing of the genome (Merchant *et al.*, 2007). Abiotic stress in *C. reinhardtii* is often linked with light availability or macro-nutrient limitation. Phosphorous and nitrogen are among the most limiting macronutrients (Reynolds, 2006). Metabolic adaptations to nitrogen limitation are well characterized in *C. reinhardtii* and are the subject of continued study (Park *et al.*, 2015). The main metabolic adaptations to nitrogen limitation include the downregulation of photosynthetic activity (Huner *et al.*, 2012; Juergens *et al.*, 2015), the accumulation of lipid droplets and the increased mobilization and allocation of internal and external nitrogen (Miller *et al.*, 2010). The downregulation of photosynthesis (reflected by fewer photosynthetic pigments such as chlorophylla) and the accumulation of neutral lipids (consisting mainly of triacylglycerols, hereafter “TAGs”) are attributed to a decreased utilization of reductant energy due to a slowdown in anabolic metabolism (Huner *et al.*, 2012). Different strains of *C. reinhardtii* do, however, display marked phenotypic differences in their responses to N-limitation (Siaut *et al.*, 2011). In a recent study, Malcom and coworkers investigated the genetic basis of growth rate variation across 18

Table 1 Functional assignment of compounds targeted by single-cell mass spectrometry and the reported population level responses in literature

Compound class and targeted peaks	Functional assignment	Change under N-limitation
Photosynthetic pigments <i>Chlorophyll a</i> , <i>Chlorophyll b</i>	Light absorbance in photosynthetic complexes.	Decrease (Miller <i>et al.</i> , 2010; Juergens <i>et al.</i> , 2015)
Thylakoid glycolipids <i>MGDG (34:7)</i> , <i>DGDG (34:6)</i> , <i>DGDG (34:5)</i> , <i>DGDG (34:3)</i> , <i>DGDG (34:2)</i>	Constituents of the thylakoid membrane embedding photosynthetic complexes.	Decrease (Li <i>et al.</i> , 2012; Yoon <i>et al.</i> , 2012)
Extraplastidic membrane lipids <i>DGTS (34:4)</i> , <i>DGTS (34:3)</i> , <i>DGTS (34:2)</i> , <i>DGTS (36:7)</i> , <i>DGTS (36:6)</i> , <i>DGTS (36:5)</i> , <i>DGTS (36:4)</i>	Extraplastidic membrane lipid that functionally replaces phosphatidylcholine. Present for example, in endoplasmic reticulum, Golgi or mitochondria.	Increase (Yang <i>et al.</i> , 2015)
Neutral storage lipids <i>TAG (50:1)</i> , <i>TAG (50:2)</i> , <i>TAG (50:3)</i> , <i>TAG (50:4)</i> , <i>TAG (50:5)</i> , <i>TAG (50:6)</i> , <i>TAG (50:7)</i> , <i>TAG (52:3)</i> , <i>TAG (52:4)</i> , <i>TAG (52:5)</i> , <i>TAG (52:7)</i> , <i>TAG (52:8)</i>	Energy storage in the form of reduced carbon. Accumulating in the form of lipid droplets in the cytoplasm.	Increase (Boyle <i>et al.</i> , 2012; Liu <i>et al.</i> , 2013; Yang <i>et al.</i> , 2015)

The number of carbons and double bonds of lipids are indicated in parentheses, for example, TAG (52:6). The detected TAG peaks can represent multiple isomers, for example, TAG (18:0/18:2/16:4), TAG (18:0/18:4/16:2) and other isomers all contribute to the peak at TAG (52:6).

strains of *C. reinhardtii* across 30 environments and found that, while genetically based phenotypic variation was constrained to only three main axes of selection (types of environmental variation), the response to nitrogen availability was one of them (Malcom *et al.*, 2015).

Here, we extended our SC-MS method to measure the impact of genetic diversity and plasticity (here defined as all phenotypic variation expressed by isoclonal populations including phenotypic heterogeneity (Ackermann, 2015)) in generating phenotypic variation under nitrogen-limited conditions. SC-MS was able to detect 26 small molecules from different pigment and lipid classes in individual cells, including chlorophylls, thylakoid lipids, membrane lipids and storage lipids (Table 1). Here, we measured the lipid composition of thousands of single cells to determine the variance and the composition of phenotypes present in different populations over time. We further investigated the correspondence between measures of population-level growth, resource limitation and single-cell phenotypes to investigate the possibility of different strategies in dealing with nitrogen limitation. To do so, we cultured the genetically diverse strain CC-1690 and two isoclonal populations isolated from CC-1690 termed ANC3 and ANC5 under N-replete and N-deplete conditions for 9 days. Population-level characterization showed differences among the populations selected for this experiment in their growth rate ($\mu_{\max}$, Supplementary Figure S1) and in their minimum nitrogen requirements (referred to here as R^* , Supplementary Figure S1). By sampling multiple time points of batch growth for SC-MS, we captured the adaptation to nitrogen depletion in the different cultures with single-cell resolution.

Materials and methods

Microbial populations and culture conditions

CC-1690 wild-type mt+ was obtained from the Chlamydomonas Resource Center, University of Minnesota (<http://www.chlamycollection.org>). The culture inoculum was grown up in sterile COMBO freshwater medium (Kilham *et al.*, 1998) lacking silicate, animal trace elements and vitamins, and then again plated onto YA agar plates to allow the formation of individual isoclonal colonies. Five isoclonal isolates were selected from agar plates by haphazardly picking five spatially isolated and distinguishable colonies and inoculating them again into liquid medium. These isoclonal isolates were designated as Ancestors 1–5 (ANC1–ANC5).

Before the single-cell MS experiment, we performed an extensive set of pilot experiments to determine the population-level growth trajectories, growth rates and minimum rates of each population in media containing a wide range of nitrate concentrations (see detailed methods in the Supplementary Information, Section 3.1). On the basis of these data (Supplementary Figures S1–S3), we selected ANC3

and ANC5 for further investigation at the single-cell level because they showed distinct population-level characteristics relative to each other and to their parental population, CC1690. In particular, ANC3 showed a high growth rate ($\mu_{\max, \text{ANC3}} = 0.96$ per day) and a higher nitrogen requirement ($R^*_{\text{ANC3}} = 1.78 \mu\text{M N}$) than CC-1690 ($\mu_{\max, \text{CC-1690}} = 0.80$ per day, $R^*_{\text{CC-1690}} = 0.27 \mu\text{M N}$). ANC5 showed a lower growth rate ($\mu_{\max, \text{ANC5}} = 0.54 \text{ d}^{-1}$) than CC-1690 and a nitrogen requirement of $R^*_{\text{ANC5}} = 0.5 \mu\text{M N}$.

Nitrate limitation experiments

Before performing the single-cell MS experiments, the wild-type strain CC-1690 and the two isoclonal isolates, ANC3 and ANC5, were acclimated and inoculated as described in Supplementary Information, Section 3.2. The batch-cultures were maintained at 20 °C, at a light intensity of $\sim 100 \mu\text{Einstein m}^{-2} \text{ s}^{-2}$, and under 100 r.p.m. continuous shaking for 9 days. We stopped the single-cell experiment on day 9 because growth trajectories from the pilot experiments had shown that the deplete cultures had all reached steady state after 8 days, at which time the replete cultures were also approaching steady state (Supplementary Figure S3). For SC-MS, the cultures were sampled 2, 4, 6 and 9 days after inoculation. For measurement of dissolved nutrients and microscopy the cultures were sampled on day 2 and 9 (details in the Supplementary Information, Section 3.3 and 3.4). The dissolved nutrient samples showed that the nitrogen concentrations were always below the 0.25 mg l^{-1} detection limit in the deplete medium, and that the concentrations of nitrogen had decreased over time in the replete environment (Supplementary Table S1).

Single-cell mass spectrometry

Single-cell MALDI mass spectrometry was performed as described recently (Krismer *et al.*, 2015). In short, slides were prepared for SC-MS as follows: on each day each culture covered a complete stainless steel slide (of 1430 spots each). Each spot was 300 μm in diameter and had a center-to-center distance of 720 μm . A layer of matrix, corresponding to $\sim 5 \text{ nl}$ of a 10 mg ml^{-1} solution of 2, 5-DHB (Sigma Aldrich, 85707) in 80% aqueous acetone, was deposited before spotting the cells. The cells were centrifuged three times at 5000 g for 5 min and resuspended in deionized water. The integrity of the cells was monitored by fluorescence microscopy throughout the sample preparation as shown in previously published work (Krismer *et al.*, 2015). To adjust the number of cells per spot for increased single-cell yield on the target, the optimal number of drops was determined for each culture using a dilution series (4×4 array of 1, 2, 3, 5, 10 drops of cell solution). The rest of the slide was covered with cells and the cells were quenched immediately using liquid nitrogen. The slide was reconstituted to room temperature in a desiccator and the lipids were

extracted from the cells and co-crystallized with the MALDI matrix. The extraction was performed in five consecutive cycles (each ~5 nl of 10 mg ml⁻¹ DHB in 80% aqueous acetone) for homogeneous lipid extraction. The whole procedure was monitored using a confocal fluorescence scanner (LS400, Tecan, Maennedorf, Switzerland). The number of cells per spot was determined on the basis of a confocal scan of the slides after quenching with liquid nitrogen. MALDI-MS parameters for the measurements on the MALDI TOF-TOF mass spectrometer (AB Sciex 5800, Sciex, Toronto, Canada) were as follows: the laser was operated at a repetition rate of 400 Hz and an intensity of 4300–4400 arbitrary units. The spectra were measured for the range of 550–1000 *m/z* with an extraction delay time of 200 ns, exhaustively ablating each spot using a spiral pattern. The absence of MS and fluorescence signals in empty spots ensures that there is no cross-contamination, and spots with multiple cells were discarded before data analysis. The details on spectral processing and peak assignment of the mass spectra can be found in the Supplementary Information, Section 3.5 and 3.6. A table with peak assignments can be found in Supplementary Table S2. A more detailed description of the SC-MS method can be found in (Krüger *et al.*, 2015). Owing to technical problems during the spotting, only very few single cells of ANC5 culture were successfully measured on days 4 and 6.

Data analysis for single-cell mass spectrometry

A principal component analysis (PCA) on the data set, including all populations, all nitrogen conditions and all days of the time series, was performed (princomp in R 3.2.4 Revised). A detailed procedure for the handling of the data (including spectral processing), submitted to PCA analysis can be found in Supplementary Figure S7. The recorded peak areas span three orders of magnitude thus the dataset was square root transformed. We used ggplot2 function including the contour plot tool density2d for visualization of the PCA (Wickham, 2009). The first five principal components (PCs) explain 91.8% of the observed variance in the data set (see Supplementary Figure S8). The PC1 (49.5% of variance explained) shows all-negative signs. The fact that all PC loadings have the same sign indicates that this PC does not refer to relative but to absolute intensities, which is underlined by the fact that the loadings closely resemble the average intensity of the peak in the experiment (see Supplementary Figure S9). In MALDI mass spectra, only relative abundances hold quantitative information, thus we restricted our interpretation on the other PCs showing loadings with opposite signs. This is especially relevant in single-cell analysis, as there is no option for introducing an internal standard and there is no single compound that is present in equal amounts in each cell. The PC2 explains 26.0% of the observed

variance and segregates between photosynthetic pigments and the extra-plastidic Diacylglyceryltrimethylhomo-Ser lipids (hereafter 'DGTS', Supplementary Figure S9). PC3 explains 8.2% of the observed variance and segregates cells that detect high levels of TAG storage lipids. PC4 explains 4.2% of the observed variance and segregates between DGTS lipids of different fatty acid composition. The details on non-parametric testing can be found in Supplementary Material, Section 3.7.

On the basis of plots of cellular phenotypes on PC2 and PC3, we assigned the cells to one of three apparent phenotypic categories. On the basis of the cut-offs of the PC scores, we defined three categorical phenotypes as 'high chlorophyll' (PC3 < 5, PC2 > 0), 'high membrane lipid' (PC3 < 5, PC2 < 0) and 'high storage lipid' (PC3 > 5). The absolute numbers of cells in each phenotypic class can be found in Supplementary Table S3. Contingency table analysis of Supplementary Table S3 by means of the Fisher's exact test was performed using Myfisher23.m function (Cardillo, 2007). The details on the bivariate data analysis can be found in the Supplementary Material, Section 3.8.

To compare the whole range of phenotypic variation observed in the isoclonal populations to that observed in the genetically diverse population, we calculated an index of multidimensional individual-level trait variation, called 'Trait Onion Peeling' or TOP (see Fontana *et al.*, 2016 for detailed description and methods). For this analysis, we were interested in the total amount of multidimensional phenotypic space covered by a population in a given treatment over the course of the whole experiment, and so we pooled all of the cells in each population × nutrient combination over time. To compare samples of equal size for each treatment, we performed a bootstrap resampling of 100 cells from each population × nutrient combination 1000 times and calculated the TOP index on the PC2 and PC3 scores of the cells in each sample. We then compared the TOP index for each bootstrapped isoclonal population with the TOP index of a bootstrapped CC1690 population, and using the 1000 repeated bootstraps, we calculated the probability that a difference in trait variation between the isoclonal populations and the genetically diverse was greater than or less than zero.

Results

Phenotypic differences among populations and treatments over time

To characterize important phenotypic differences among the three populations, CC-1690, ANC3 and ANC5 in response to nitrogen-depletion treatment, we used PCA to decompose the variation in the mass spectra. The SC-MS values report differences in relative lipid and pigment composition of the single cells. PC1 refers to the absolute intensities of peaks in the mass spectra that cannot be assessed

Figure 1 Contour plots of the PCA performed on the single-cell mass spectra. PC2 segregates between ‘high chlorophyll’ and ‘high membrane lipid’ cells corresponding to high and low PC2 values, respectively. PC3 additionally segregates ‘high storage lipid’ cells. There is a general trend of all N-depleted populations from ‘high chlorophyll’ via ‘high membrane lipid’ towards ‘high storage lipid’.

quantitatively using our MALDI-MS method (see the ‘Materials and methods’ section). PC2, however, separated between chlorophylls and DGTS while PC3 additionally separated between TAGs (Figure 1; for the PC loadings, see Supplementary Figure S9). Based on this analysis, we defined three phenotypes called ‘high chlorophyll’ (PC2 > 0 and PC3 < 5), ‘high membrane lipid’ (PC2 > 0 and PC3 < 5) and ‘high storage lipid’ (PC3 > 5; see Supplementary Table S3). For exemplary spectra of the three phenotypes, see Figure 2b. For PC scores of the respective cells see Supplementary Figure S10. The ‘high chlorophyll’ phenotype was ubiquitous in N-replete cultures and especially prominent on day 2 (for example, 97.4% of cells measured in N-replete CC-1690). Fisher’s exact test showed that in deplete cultures there was no significant difference between the phenotype frequencies in CC-1690 and ANC3 on day 2 ($P = 0.20$) while CC-1690 and ANC5 differed significantly ($P = 0.008$). With increasing nitrogen limitation ‘high chlorophyll’ phenotypes made up a decreasing fraction of the population (see green circles in Figure 2a) while the fraction of ‘high membrane lipid’ phenotypes increases (Table 1). Focusing on the most abundant chlorophyll (chlorophyll a) and the most abundant extraplastidic membrane lipid in the spectra (DGTS (34:3)), a bivariate analysis revealed a

marked transition between ‘high chlorophyll’ stages on day 2 and ‘high membrane lipid’ on day 9 in N-deplete cultures as can be seen in Figure 3. Remarkably, CC-1690 shows a distinct bimodal distribution including both spectra high in chlorophyll a and high in DGTS (34:3) on day 9.

The three populations also differed in the timing of the onset of the ‘high storage lipid’ phenotypes (Table 1) in N-depleted cultures (Figure 1 and Figure 2a). In CC-1690, we detected ‘high storage lipid’ phenotypes throughout the time series (12.5–36.3% of the cells in the population); whereas in ANC3, only small fractions of the populations showed ‘high storage lipid’ phenotype until day 6 (1.6% on day 2, 4.8% on day 6, see also Supplementary Table S3). ANC5 showed a very small fraction of ‘high storage lipid’ phenotypes on day 4 (3.9%) and on day 6 (0.6%). Highest frequencies of ‘high storage lipid’ phenotypes for both isoclonal populations were found on day 9. ANC3 contained almost 40% of cells with a ‘high storage lipid’ phenotype; whereas ANC5 contained the lowest level of ‘high storage lipid’ phenotype cells (14.8%) in comparison with the other two populations. Fisher’s exact test showed that under deplete conditions, CC-1690 differed significantly both from ANC3 ($P = 0.00008$) and from ANC5 ($P \approx 0$)

on day 9. In CC-1690, phenotype frequencies were not significantly different between day 6 and day 9 ($P=0.91$). Furthermore, in CC-1690 was the first culture to respond to nitrogen stress with 'high storage lipid' phenotypes, but also the population

that was able to retain the highest fraction of 'high chlorophyll' cells until day 9 (15.6% in CC-1690, 3.6% ANC3 and 3.2% in ANC5). Furthermore, CC-1690 N-deplete and N-replete cultures displayed greater phenotypic similarity on day 9 than ANC3 and ANC5, as shown by the high overlap of contour plots of CC-1690N-replete and N-deplete cultures in Figure 1.

Parallel chlorophyll measurements using microscopy

As SC-MS only reports relative compound levels and we cannot assume one of the compounds to be present in equal amounts in all the single cells measured, we aimed to confirm the trends in chlorophyll levels that we observed using SC-MS using fluorescence microscopy. As per our MS data, the fluorescence microscopy showed that on day 2, the N-replete and N-deplete cultures tended to have had comparable chlorophyll levels; whereas on day 9, replete cultures were relatively much higher in chlorophyll than deplete cultures (Figure 4 and Supplementary Table S4). Fluorescence microscopy also showed that ANC5 cells had relatively low absolute levels of chlorophyll on day 9. ANC3 still shows the highest absolute chlorophyll intensities of all populations under N-deplete conditions on day 9 (Figure 4 and Supplementary Table S4).

Comparisons of pooled trait variation

The TOP index is an estimate of the cumulative area of multidimensional trait space that is covered by individuals of a population. We used bootstrapping to compare the temporally pooled trait variation between isoclonal and diverse populations of equal size (100 cells). The analysis showed that the bootstrapped distribution of estimated trait variation (TOP) for ANC3 was largely overlapping with that of CC1690, regardless of nitrogen treatment (Supplementary Figures S11a and b). By comparison, the distribution of ANC5 TOP index showed lower levels of trait variation than CC1690 in both the deplete (mean difference = -180.79, Supplementary Figure S11c) and the replete (mean difference = -220.51, Supplementary Figure S11d) environments. However, in all the cases, the bootstrap probabilities that the clones had greater or lower individual-level trait variation than the popula-

Figure 2 (a) Schematic of the relative phenotypic composition of the populations over time. Each circle represents a 5% fraction of cells within the population (for more details, see also Supplementary Table S3). Green circles represent the 'high chlorophyll', blue circles the 'high membrane lipid' and red circles the 'high storage lipid' phenotypes. (b) Exemplary single-cell mass spectra of the three phenotypes 'high chlorophyll', 'high membrane lipid' and 'high storage lipid'. The spectra were background subtracted using a proximate empty spot mass spectrum. 'High chlorophyll' and 'high membrane lipid' cells are from replete ANC3 culture on day 9. 'High storage lipid' cell is from deplete ANC3 culture on day 9.

Figure 3 Violin plots of the log-transformed ratio of the peak area of DGTS (34:4) and chlorophyll a. Histograms are smoothed using a kernel density estimation. Red crosses indicate population averages. Replete ANC5 cultures on day 4 and day 6 showed too few successful measurements (less than 10) to be included in the analysis.

tion were all <0.95 (reported in Supplementary Figure S11), and were therefore not statistically significant at the $P=0.05$ critical threshold.

We were interested first and foremost in the multidimensional trait variation of the populations, but we also investigated differences in temporally pooled trait variance along individual PC axes for each populations $\times$ nitrate treatment combination. ANC3 did not show significant differences from CC1690 along PC2, but it showed significantly lower variance than CC1690 in the replete environment, and significantly higher variance in the deplete environment along PC3 (Supplementary Table S5, Supplementary Figure S11 and S12). By contrast, ANC5 showed lower variance than CC1690 in all scenarios, except along PC2 in the deplete environment, where it had significantly higher trait variance (Supplementary Table S5, Supplementary Figure S11 and S12).

Altogether, this suggests that both isoclonal populations had equal or lower phenotypic variation than the genetically diverse population in multidimensional trait space, but that they were able to display greater phenotypic variance in individual trait dimensions under nitrogen stress (Supplementary Figure S11): ANC3 had greater variance along

PC2 and ANC5 had greater variance along PC3 (Supplementary Figure S12).

Discussion

The single-cell phenotype data collected here are the first of their kind in terms of complexity and extent. The depth of information collected from each cell revealed marked cell-to-cell variation within each culture at each time point. At the population level, the measurements followed the general trends reported in the literature with regards to *C. reinhardtii*'s responses to nitrogen depletion, including the decrease in chlorophyll, an increase in DGTS content, and the accumulation of TAGs (Juergens *et al.*, 2015; Park *et al.*, 2015; Yang *et al.*, 2015). Following the phenotypic composition of the populations at the single-cell level over time renders a dynamic picture of changes in phenotypic variation within populations of *C. reinhardtii* in response to this well-studied nutrient stressor. Furthermore, it provides information about the amount of phenotypic variation generated by plasticity alone versus that generated by both plasticity and genetic diversity together.

Figure 4 Chlorophyll fluorescence levels in relation to cell size determined using fluorescence microscopy.

Our results show that genetic diversity tends to result in greater multidimensional phenotypic variation among cells within a population over time during N-limitation than plasticity alone (Figure 2a, Supplementary Figure S11). Phenotypic variation (that is, the expression of all three phenotypes: ‘high chlorophyll’, ‘high membrane lipid’ and ‘high storage lipid’) in the genetically diverse population is maintained over the time course of the experiment, while the isoclonal populations show more synchronicity in their response to N-limitation. It has been reported that the accumulation of high levels of TAG are a mechanism to alleviate oxidative stress concurrent with the slowdown of anabolic metabolism upon N-limitation (Huner *et al.*, 2012). We therefore attribute the ‘high storage lipid’ phenotype to cells that experience high N-limitation stress. Although both isoclonal populations showed ‘high storage lipid’ phenotypes almost exclusively on day 9, the diverse population already showed cells with ‘high storage lipid’ phenotype within the first 6 days of the experiment. By contrast, CC-1690 also included the highest fraction of cells with a ‘high chlorophyll’ phenotype on day 9. It should be noted that the level of a certain metabolite or biopolymer (including chlorophyll) in a single cell is the result of both its biosynthesis and degradation. Nevertheless, the presence of ‘high chlorophyll’ cells in replete CC-1690 on day 9 might be an indication of cells that are little affected by the nitrogen-limitation, and is consistent with its relatively low nitrogen requirement (N^*) and high maximum specific growth rate ($\mu_{\max}$, Supplementary Figures S1 and S2). The functional interpretation of the ‘high membrane lipid’ phenotype is more difficult because extraplastidic membranes are involved in a variety of metabolic processes in the ER, the Golgi apparatus and the plasma membrane (Table 1). Despite the

clear change in DGTS/chlorophyll, a ratio in N-deplete cultures (Figure 3), the abundance of the ‘high membrane lipid’ phenotype and the variation of DGTS/chlorophyll a ratio in replete cultures suggests that the increase of ‘high membrane lipid’ phenotypes is not only a response to nitrogen limitation, but is also part of the phenotypic variation in the N-replete environment.

SC-MS suggested that ANC3 underwent a transition from ‘high chlorophyll’ to ‘high membrane lipid’ phenotypes early on (70% ‘high membrane lipid’, 30% ‘high chlorophyll’ on day 4). Fluorescence microscopy of the same population revealed that ANC3 still shows highest chlorophyll levels on day 9 compared with CC-1690 and ANC5 (Figure 4, Supplementary Table S4). This suggests that downregulation of photosynthesis under N-limitation might be less pronounced in ANC3. However, ANC3 also showed a relatively high fraction of ‘high storage lipid cells’ on day 9, indicating high levels of nitrogen limitation. This is also in agreement with the significantly higher nitrogen requirement (N^*) of ANC3 compared with CC-1690 (Supplementary Figure S1). By contrast, ANC5 showed lowest chlorophyll values per cell compared with both CC-1690 and ANC3 on day 9 in fluorescence microscopy. However, the fewer ‘high storage lipid’ cells (14.8%) compared with both ANC3 and CC-1690 suggest an adaptive downregulation of photosynthesis. Population measures showed that ANC5 has a significantly lower population-level growth rate, which further supports the hypothesis that ANC5 shows conservative growth behavior beneficial in nutrient-limited environments. Based on these responses of the isoclonal populations, it would stand to reason that they represent different strategies in coping with nutrient limitation. By keeping photosynthetic activity low, ANC5 may

decrease its risk of running into oxidative stress upon intensification of nitrogen limitation. On the other hand, in keeping high amounts of chlorophyll, ANC3 may be able to utilize available nitrogen more quickly when it is supplied at a higher concentration in the environment (see Figure 4 and Supplementary Table S4). This is supported by a faster maximal population growth rate (Supplementary Figure S2), particularly in replete conditions (Supplementary Figure S2).

When we compared the full range of phenotypic variation across populations over time, we found that the genetically diverse population tended to have an equal or greater multidimensional phenotypic variation than the isoclonal populations, that is, the cells more fully covered the multidimensional trait space. This was despite the fact that the clones both showed greater phenotypic variance along individual trait axes under N-limitation: ANC3 showed greater variance along PC3 and ANC5 along PC2. One explanation is that plasticity in this system confers the ability to maintain high-fitness-associated phenotypes under stress (that is, N-limitation), providing phenotypic buffering, rather than promoting the expression of phenotypic variance (Reusch, 2014). Accordingly, each clone is able to plastically buffer the expression of low-fitness, highly stressed, phenotypes in one phenotypic dimension, but not the other. By contrast, the genetically diverse population, which covers more of the multidimensional phenotype space, may be phenotypically buffered to the influences of the N-limitation along both individual phenotypic axes due to genetic compensation, or the increase in the abundance of genotypes with high-fitness-associated phenotypes (Grether, 2005). This assumes that the reduced phenotypic variation along individual trait axes, but the greater coverage of phenotypic space in multiple dimensions in the genetically diverse population is adaptive. Though we did not measure the fitness of individual phenotypes, this interpretation is supported by the relatively high population-level growth rate and low nitrogen requirement of CC1690 compared with the isoclonal populations. Overall, our results suggest that genetic diversity can produce equal or greater multidimensional phenotypic variation (trait richness) under nitrogen stress as non-genetic sources, but that it can result in significantly lower phenotypic variation in response to stress along individual phenotypic dimensions.

Multidimensional phenotype data from single cells, such as those presented here, are invaluable in the study of phenotypic adaptation. The key abilities of SC-MS are that (i) multiple cellular processes can be monitored simultaneously, (ii) high-throughput analysis can render a cross-section of the phenotypic composition of populations and (iii) the method can be applied on multiple time points in an experiment, which reveals information on changes in the phenotypic composition of populations over time. Our

experiments also show the benefit and need for complementing single-cell mass spectrometric data with other phenotypic data collected from the same population and the bridging of single-cell information with population characteristics, which enhances this highly multiparametric experimental approach. Future applications of the method should also aim to use the method in an experimental context or include biological replicates for each measurement. The experiments showed here further underline that SC-MS can detect biologically functional phenotypic variation in populations, that is, differences between cells that can make a difference for the whole population. From this analysis, we learned that non-genetic multidimensional phenotypic variation tends to be more limited and synchronous over time than genetically based phenotypic variation, but the cumulative non-genetic phenotypic variance over time can be greater along individual phenotypic dimensions than that found in genetically diverse populations. The consequences of these findings for eco-evolutionary dynamics are beyond the scope of our study, but would be a fruitful avenue for future investigation.

Conflict of Interest

The authors declare no conflict of interest.

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