A multidimensional approach to the expression of phenotypic plasticity

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RESEARCH ARTICLE

A multidimensional approach to the expression of phenotypic plasticity

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Abstract

1. Phenotypic plasticity is increasingly recognized as a key element of eco-evolutionary dynamics, but it remains challenging to assess because of its multidimensional nature. Indeed, organisms live in complex environments where numerous factors can impact the phenotypic expression of traits (inter-environment axis), possess multiple traits that can influence each other's expression (inter-trait axis) and differ in their genetic background (inter-genotype axis), which can not only impact the traits' values but also their plasticity.
2. We addressed six questions related to phenotypic plasticity: (a) do different environmental gradients show similar effects on a given trait? (b) Are the effects of two environmental gradients on a trait additive? (c) Do different traits show similar plastic response to a given environmental gradient? (d) Do the (co)variances between traits vary across environmental gradients? (e) Do genotypes differ in their plastic response to a given environmental gradient? (f) Are some genotypes more plastic than others across all traits?
3. We designed a microcosm experiment using the protist *Tetrahymena thermophila* aimed at encompassing all these aspects of phenotypic plasticity. We exposed 15 distinct genotypes to 25 combinations of temperature and nutrient availability and assessed the plasticity of five phenotypic traits.
4. Our results show strong differences in the plastic response depending on the environmental gradient, not only regarding the shape of the reaction norm of the different traits tested, but also in the overall plasticity of the organisms. We did not find any covariance between traits that was consistent across all environments.
5. Overall, our results suggest independent impacts of the environmental dimension considered on the observed plastic response. These results underline potential difficulties in generalizing findings about plasticity to all environments and all traits.

KEYWORDS

controlled microcosms, genotype × environment experiment, G-matrix, phenotypic plasticity, phenotypic syndrome, *Tetrahymena thermophila*, variance partitioning

1 | INTRODUCTION

Phenotypic plasticity is increasingly recognized as a critical factor in mediating eco-evolutionary processes (Carroll, Hendry, Reznick, & Fox, 2007; DeWitt & Scheiner, 2004; Forsman, 2015; Hendry, 2015; Pigliucci, 2001). Research over the past decades has revealed the ubiquity of plasticity (Miner, Sultan, Morgan, Padilla, & Relyea, 2005; Palacio-López, Beckage, Scheiner, & Molofsky, 2015; Pigliucci, 2001; Price, Qvarnström, & Irwin, 2003; West-Eberhard, 2003) and identified the developmental (Emlen & Nijhout, 2000; Hoverman & Relyea, 2007; Murren et al., 2015), genetic (Callahan, Dhanoolal, & Ungerer, 2005; DeWitt & Scheiner, 2004; Van Kleunen & Fischer, 2005) and epigenetic (Auge, Leverett, Edwards, & Donohue, 2017; Ledón-Rettig, Richards, & Martin, 2012; Smith & Ritchie, 2013) processes underlying plasticity. Plasticity occurs when a given genotype produces different phenotypes in response to external factors, which may increase its short-term success when confronted with varying environments (DeWitt & Scheiner, 2004; Pigliucci, 2001). The value of such plasticity ranges from buffering the impacts of changing environmental conditions (Ghalambor, McKay, Carroll, & Reznick, 2007; Reed, Waples, Schindler, Hard, & Kinnison, 2010) to facilitating colonization of new habitats (Geng et al., 2016; Richards, Bossdorf, Muth, Gurevitch, & Pigliucci, 2006; Yeh & Price, 2004), and helping to cross 'adaptive valleys' during population divergence and speciation (Pfennig et al., 2010; van Snick Gray & Stauffer, 2004).

Much of the early research on phenotypic plasticity has focused on the response of traits to a single environmental gradient (e.g. Bruno & Edmunds, 1997; Denver, Mirhadi, & Phillips, 1998; Weider & Pijanowska, 1993). Yet, natural environments are composed of a multitude of interacting factors potentially influencing the plastic expression of traits. For example, the growth rate of the seed beetle *Callosobruchus maculatus* varies with both temperature and the rearing host (Stillwell, Wallin, Hitchcock, & Fox, 2007), and the laying date of great tit *Parus major* varies with both temperature and daylength (Gienapp, Väisänen, & Brommer, 2010). However, our understanding of how multiple environmental gradients influence reaction norms remains limited (Westneat, Potts, Sasser, & Shaffer, 2019). The effect of two environmental dimensions could be additive, antagonistic, synergistic or complementary. Determining how multiple environments influence plasticity is challenging because it requires measures of independent and joint effects of factors on the same genotype through fully factorial experimental designs.

Studies concerning multivariate plasticity focus on the interaction between multiple phenotypic traits and how each trait affects phenotypic plasticity in the other trait (Laughlin & Messier, 2015; Westneat et al., 2019). Indeed, organisms are mosaics of traits interacting to influence fitness (Dochtermann & Dingemanse, 2013; Legrand et al., 2016; Sih, Bell, & Johnson, 2004), which raises several questions. Firstly, will a single environmental factor cause plastic changes on several traits, or will traits be independently influenced by different factors? Secondly, for a given environmental factor, will the change in each trait be in a similar direction and magnitude, and how will the covariances between traits be impacted? While considerable information has accumulated on the shapes of reaction norms for a single trait measured along a single environmental gradient, we still need to determine if reaction norms are generalizable across traits and/or environmental factors.

Moreover, genotypic variation in the shape of reaction norms further increases the complexity of understanding the multidimensional nature of phenotypic plasticity. For instance, differences in reaction norms occur between populations of seed beetles from Burkina Faso and South India in their plastic response to temperature (Stillwell et al., 2007) and between populations of great tits from the Netherlands and the United Kingdom (Charmantier et al., 2008; Husby et al., 2010). To what extent reaction norms are similar across genotypes, similar across several traits of a given genotype, and whether some genotypes display more phenotypic plasticity on average across all traits than other genotypes is largely unexplored.

Phenotypic plasticity is therefore a complex phenomenon involving different kinds of multidimensionalities, usually addressed within separate studies. We identified three different axes along which this multidimensionality deserves deeper investigation (Figure 1): (a) the inter-environment axis, concerning interactions between the response to multiple environmental factors (Westneat et al., 2019), (b) the inter-trait axis, concerning the interactions between the responses of multiple traits to the same environmental factors (Laughlin & Messier, 2015) and (c) the inter-genotype axis, concerning the relationship between the genetic background of organisms and their plastic response to environmental factors (Pigliucci, Murren, & Schlichting, 2006; Richards et al., 2006).

In this study, we investigated the complex interplay between these three axes in the expression of phenotypic plasticity. We used a series of controlled fully factorial microcosm experiments in 15 clonal genotypes of a ciliated protist *Tetrahymena thermophila*, where we manipulated temperature and nutrient concentration and measured the expression of five phenotypic traits linked to fitness, morphology and movement. These two environmental factors were chosen as they are both likely to vary over space and time in the natural habitat (freshwater bodies), where *T. thermophila* feeds on bacteria. Furthermore, with increasing temperature, metabolism accelerates leading to higher resource demand (Gerhard, Koussoroplis, Hillebrand, & Striebel, 2019). This effect can be compensated by higher nutrient supply. The existence of phenotypic plasticity and its variation among genotypes have been shown previously in this species for various morphological, behavioural and fitness traits, but only along single environmental gradients, for example, density dependence of dispersal (Pennekamp, Mitchell, Chaine, & Schtickzelle, 2014) and thermal performance (Jacob et al., 2018). Here, we examined plasticity in morphological, behavioural and fitness traits along two different environmental gradients, for each genetic line. We aimed at answering six questions (Figure 1): (1) Do different environmental gradients show similar effects on a given trait? (2) Are the effects of two environmental gradients on a trait additive? (3) Do different traits show similar plastic responses to a given environmental gradient? (4) Do the (co)variances between traits vary across environmental gradients? (5) Do genotypes differ

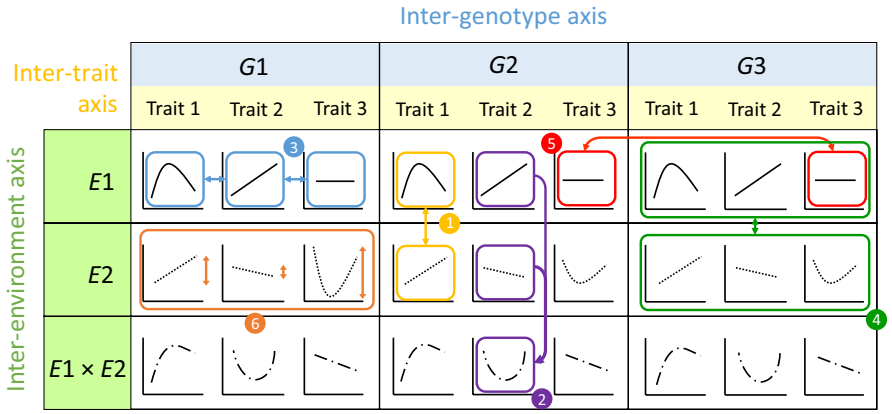


FIGURE 1 Schematic representation of the six different questions considered in this study and their relationship to the three identified multidimensionality axes. We have represented hypothetical reaction norms of three traits for three genotypes (*G*1, *G*2 and *G*3) in two environmental gradients (*E*1 and *E*2) and their interaction (*E*1 × *E*2). We asked: (1) if different environmental gradients show similar effects on the plasticity of a given trait, (2) if the effects of two environmental gradients on a trait are additive, (3) if different traits show similar plastic response to a given environmental gradient, (4) if (co)variances between traits vary across environmental gradients, (5) if there are differences between genotypes in their plastic response to a given environmental gradient and (6) if some genotypes are more plastic than others across all traits. Each question is illustrated with a single example comparison only

in their plastic response to a given environmental gradient? (6) Are some genotypes more plastic than others across all traits.

2 | MATERIALS AND METHODS

2.1 | Strains and cultures

We used 15 isogenic lines (referred to as 'genotypes') of *T. thermophila*, a free-living unicellular eukaryote. This species has been used for decades as a model species in molecular biology and physiology and more recently in evolutionary ecology (Chaine, Schtickzelle, Polard, Huet, & Clobert, 2010; Fjerdingstad, Schtickzelle, Manhes, Gutierrez, & Clobert, 2007; Jacob, Clobert, Legrand, Schtickzelle, & Chaine, 2016; Jacob et al., 2017, 2018; Pennekamp et al., 2014; Schtickzelle, Fjerdingstad, Chaine, & Clobert, 2009). The genotypes used for this study were either isolated from natural populations across different sites in North America or created by subsequent crossings between genotypes in the laboratory (see Table S1). They were cultivated in light-controlled incubators (14 hr light/10 hr dark-cycle at 27°C) in an axenic medium (PPYE 1x: 2% Proteose Peptone and 0.2% Yeast Extract [Becton Dickinson] diluted in ultrapure water [Altermatt et al., 2015]). Culture stocks were renewed every 10 days by inoculating a 2 ml sample of fresh medium with 100 µl of culture and maintained in 2 ml 24-well plates (CELLSTAR ref. 662160; Greiner BioOne). All manipulations of axenic cultures were conducted under sterile conditions in a laminar flow hood (Ultrasafe 218 S).

2.2 | Experimental design

We performed a Genotype-by-Environment ($G \times E$) experiment, where the Genotype factor was made up of the 15 clonal genotypes of *T. thermophila*. The Environment factor was all 25 possible combinations of five temperature (*T*) levels (15, 21, 27, 33 or 39°C), and five nutrient concentration (*N*) levels, as dilutions of the original PPYE 1x medium (0x, 0.125x, 0.25x, 0.5x or 1x). The ranges of temperature and of nutrient concentration considered was the largest possible, given technical and biological constraints. Indeed, higher temperatures lead to massive mortality, while lower temperature and higher nutrient concentration would strongly hinder cell movement and metabolism. The complete experimental design then comprised 375 $G \times E \times E$ combinations ($15G \times 5T \times 5N$). The whole experiment was replicated three times for each combination and a total of 1,125 cultures.

One mother culture was initiated per genotype before each repetition of the experiment, by inoculating 5 ml of PPYE 1x with 500 µl of monoculture of a given genotype and incubated in the standard culture conditions (27°C, PPYE 1x) for 2 days. Each of these 15 '*G*' mother cultures was then transferred in 100 ml of fresh PPYE 1x, conserved for five additional days in the same conditions to reach sufficient cell concentration for the experiment. Then, they were carefully homogenized and aliquoted into five smaller tubes, from which the old medium was removed by aspiration after 5 min of centrifugation at 250 g and replaced by 10 ml of fresh PPYE nutrient, for each of the five different nutrient concentration levels. Finally, each of these 75 '$G \times N$' culture was diluted to a standardized density of 300 kcell/ml with fresh PPYE medium at the same concentration, split into five 4 ml cultures, each incubated at one of the five temperatures tested to start the experiment, giving 375 '$G \times N \times T$' cultures. Data were collected at two times during the experiment on these cultures. The cell density was measured after 24 hr, as a proxy for population growth. Then, each culture was standardized at a density of 100 kcell/ml through dilution into fresh medium and re-incubated with their respective nutrient concentration and temperature for one additional hour. The main reason to proceed in this way was to avoid a potential bias in the measure of movement

behaviour arising when cell density is too high. In such conditions, 'collisions' between swimming cells blur cell identity during tracking, cutting each movement trajectory into shorter and straighter bits, biasing speed and linearity. On the contrary, morphology is not largely affected over such a short time (1 hr) given that temperature and nutrient concentration conditions were unchanged.

Five metrics related to morphology, behaviour and fitness were quantified during the experiment using our standardized digital picture workflow (Pennekamp et al., 2014; Pennekamp & Schtickzelle, 2013; Pennekamp, Schtickzelle, & Petchey, 2015): cell density, cell size, cell shape, movement speed and movement linearity. For each culture, five 10 μl samples were extracted after culture homogenization and each loaded into a chamber of a counting slide (Precision cell, Kima, Italy). The first three variables were obtained by analysing the pictures taken of each chamber under a dark field microscope. The density was estimated from the number of cells counted after 24 hr. The cell size as the area of the cross section in μm^2. The cell shape as the major/minor axis ratio of a fitted ellipse, the minimal shape value being 1 for a perfectly round cell. The last two variables concerning movement were obtained from one video taken for a randomly chosen chamber among the five available for each culture. The videos were analysed using the BEMOVI R package (Pennekamp et al., 2015) to reconstruct movement trajectories and obtain movement speed and linearity. Linearity was quantified as the net-to-gross distance ratio, net and gross distances being the Euclidean distance between the starting and arrival points and the effective length of the trajectory respectively. The maximal linearity value of 1 then indicates a perfectly straight path.

2.3 | Analysis of the results

The statistical analyses of the experimental results were all performed using the R software (R Core Team, 2018). The response variables (i.e. the five phenotypic traits) were analysed using an ANOVA for variance partitioning and a random regression-mixed model for describing the mean reaction norm. First, we used an ANOVA model to quantify how the variance in the trait values is explained by the temperature (*T*), the nutrient concentration (*N*), the genotype (*G*) and their interactions noted $T \times N$, $G \times T$, $G \times N$ and $G \times T \times N$. To account for the difference in the number of levels of each environmental factor (5) and of genotypes (15), we performed the variance partitioning for every possible subset of five genotypes among the 15. For each term, we estimated the respective effect size of each factor and their interaction using the η^2 metric (Fritz, Morris, & Richler, 2012), and then we computed the average value of η^2 over all these subsets as metrics of trait variance.

Reaction norms, that is., average trait values over the two-dimensional space of temperature and nutrient concentration, were assessed using quadratic random regression-mixed model analyses (Morrissey & Liefting, 2016) using the LME4 package (Bates, Maechler, Bolker, & Walker, 2014). For each trait, the mixed model took the following form:

$$z_{i,G} = a + b_1 T_i + b_2 T_i^2 + b_3 N_i + b_4 N_i^2 + b_5 T_i N_i + c + d_{1,G} T_i + d_{2,G} T_i^2 + d_{3,G} N_i + d_{4,G} N_i^2 + d_{5,G} T_i N_i + e_i,$$

with $z_{i,G}$ the value of the trait for experimental replicate *i* for genotype *G* exposed to temperature T_i and nutrient concentration N_i. To limit the size of the covariance matrix used to estimate random effects and to ensure the convergence of the models, we limited the interactions to the linear terms (T_i and N_i). The coefficients a, b_1, b_2, b_3, b_4 and b_5 were used to estimate the reaction norm, while accounting for potential nonlinear relationships between the trait values and the environment and interactions between the environmental gradients. *T* and *N* values were each centred and standardized prior to the analysis to ensure independence between linear and quadratic terms as well as comparability of their size effect despite being expressed in different units (Schielzeth, 2010).

For each of the 5×5 environmental combinations, a *G*-matrix was computed to describe the (co)variances between the values of each trait across all genotypes. Each *G*-matrix included the variance of each trait on its diagonal, and the covariances between the five traits off diagonal. Since *G*-matrices are symmetrical, the covariances in the upper and lower triangles were identical and each matrix included 15 unique values: five variances and 10 covariances. To account for differences in the ranges of the trait values, a mean standardization was performed by dividing the raw values by the mean value for each environmental condition (Delahaie et al., 2017; Kirkpatrick, 2009). We computed the effective number of dimensions of each *G*-matrix n_D, which can theoretically vary between 1 (the *G*-matrix is singular) to the number of traits (the *G*-matrix is full rank, here 5) and decreases when fewer dimensions are enough to describe the full information content in the *G*-matrix. This happens because of existing covariation between traits and/or heterogeneity in the level of variance in the different traits (Kirkpatrick, 2009). To disentangle the relative importance of these two aspects in giving low n_D values, we computed an extra measure n'_D, corresponding to n_D obtained in a set of 25 *G*-matrices with variances identical to the observed ones but covariances forced to 0. This n'_D value sets the maximum number of effective dimensions in the absence of covariance between traits but taking heterogeneous trait variances into account. Then, we computed *r* as the reduction in effective number of dimensions because of covariances among traits as follows:

$$r = (n_D - n'_D)/(n'_D - 1).$$

The value of *r* varied between 0 if the dimensionality reduction was entirely depending on heterogeneous variances, and −1 if it was entirely depending on covariances.

Finally, the coefficient of variation of each trait was computed for each genotype across *T* and across *N* as a proxy for the magnitude of their plasticity. The genotypes were ranked according to these coefficients (from 1 for the least plastic to 15 for the most plastic), and these ranks were compared using Kruskal–Wallis tests to identify differences in the average plasticity of genotypes over the five measured traits.

3 | RESULTS

3.1 | Do different environmental gradients show similar effects on a given trait?

The variance partitioning based on an ANOVA model allowed us to assess the relative importance of temperature (*T*) and nutrient concentration (*N*) on the variance of each of the five traits, for the levels of *T* and *N* considered in the experiment (Figure 2). The results show clear differences in the amount of variance in the traits that were affected by the two environmental gradients. For instance, the shape of cells was only strongly impacted by *T* but not by *N*, while the size of the cells was only strongly impacted by *N* but not by *T*. Density, a measure of demography (population growth) given that initial densities were normalized, was greatly affected by both *T* and *N*, whereas movement traits (speed and linearity) were impacted only slightly by *T* and *N*.

3.2 | Are the effects of two environmental gradients on a trait additive?

The interaction between *T* and *N* was systematically low for every trait (Figure 2), indicating that the impacts of the two environmental gradients on the response were mostly additive. This is confirmed by the predicted mean reaction norms drawn across all genotypes (Figure 3, right), which show largely independent responses to the two environmental gradients.

3.3 | Do different traits show similar plastic responses to a given environmental gradient?

While some traits showed similar plastic responses to a given environmental gradient, neither manipulated environmental feature influenced all traits in the same way. Density, cell shape and linearity were consistently influenced by *T*. Interestingly, reaction norms of these traits were all quadratic (Figure 3) and all reached their inflection points (maximum density, maximally elongated shape and minimal linearity) for intermediate values of *T*. Density and cell size were consistently plastic to *N* (Figure 2). They exhibited a strong reduction of trait values at the lowest concentration (0x) compared to the other ones (Figure 3).

3.4 | Do the (co)variances between traits vary across environmental gradients?

There were large differences between the variances of the different traits, for all the *G*-matrices (Figure 4a,b). The variance in shape was systematically low for all environments, while variances in speed and in linearity were on average higher, and tended to increase with temperature (Figure 4a). The impact of nutrient concentration on

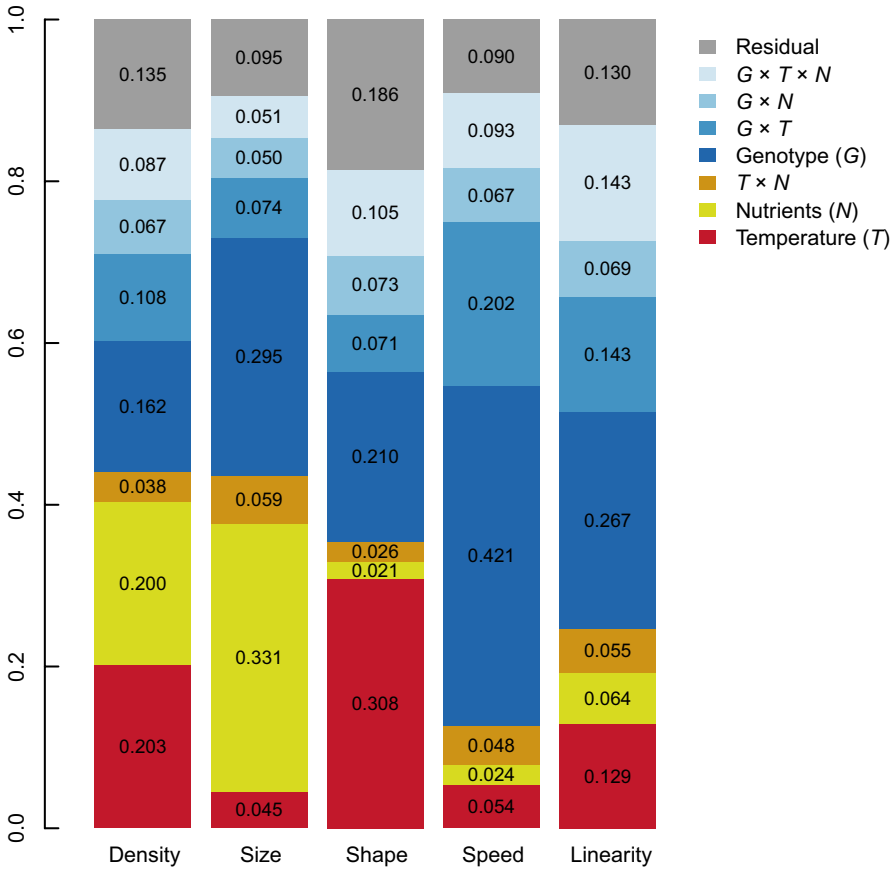


FIGURE 2 Proportion of variance (η^2) explained by the different factors using an ANOVA model. To accommodate differences in number of levels between temperature (5), nutrient concentration (5) and genotypes (15), the ANOVA was fitted independently to all possible subsets of five genotypes and η^2 averages reported here

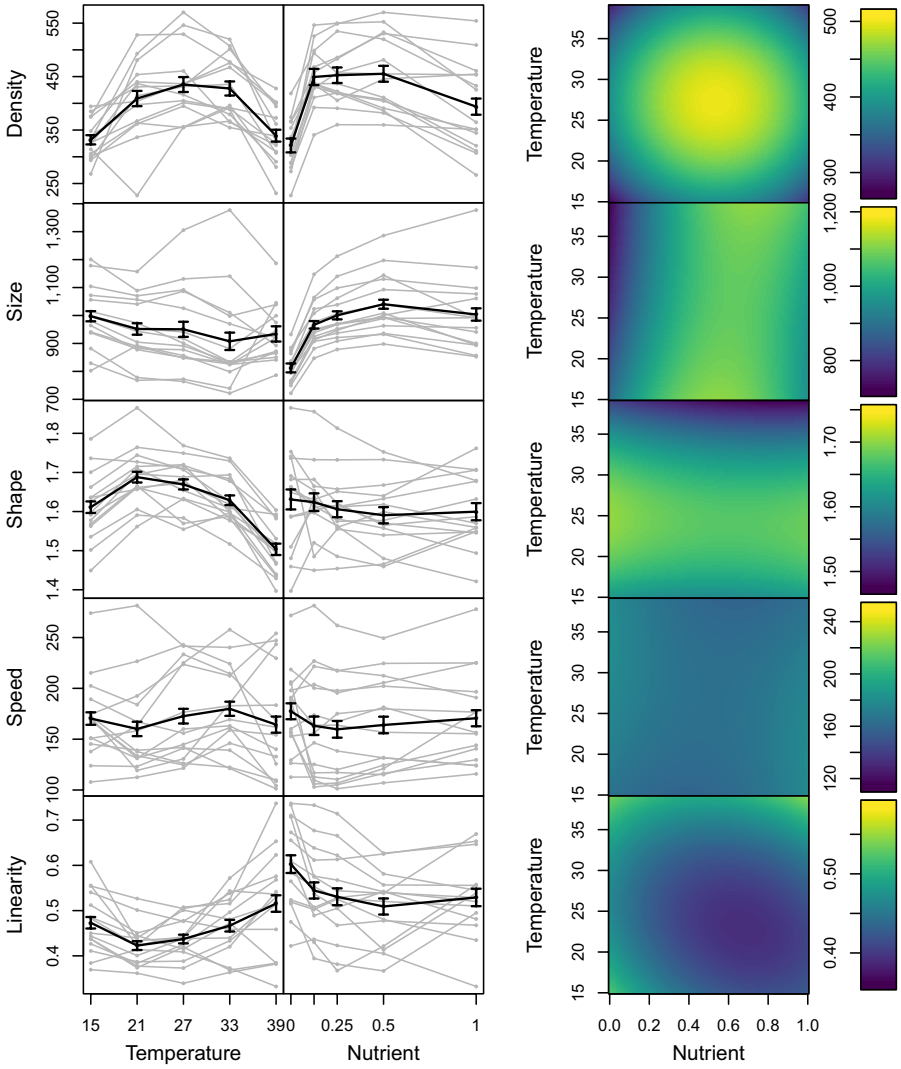


FIGURE 3 Reaction norms of the five traits according to the two environmental gradients (temperature and nutrient concentration). (Left) Observed values for each genotype (grey lines, averaged over three replicates) and averaged over all genotypes (black line ± 2 *SE*) for each environmental gradient separately. (Right) Predicted mean reaction norms across the $T \times N$ environmental space from the random regression-mixed model

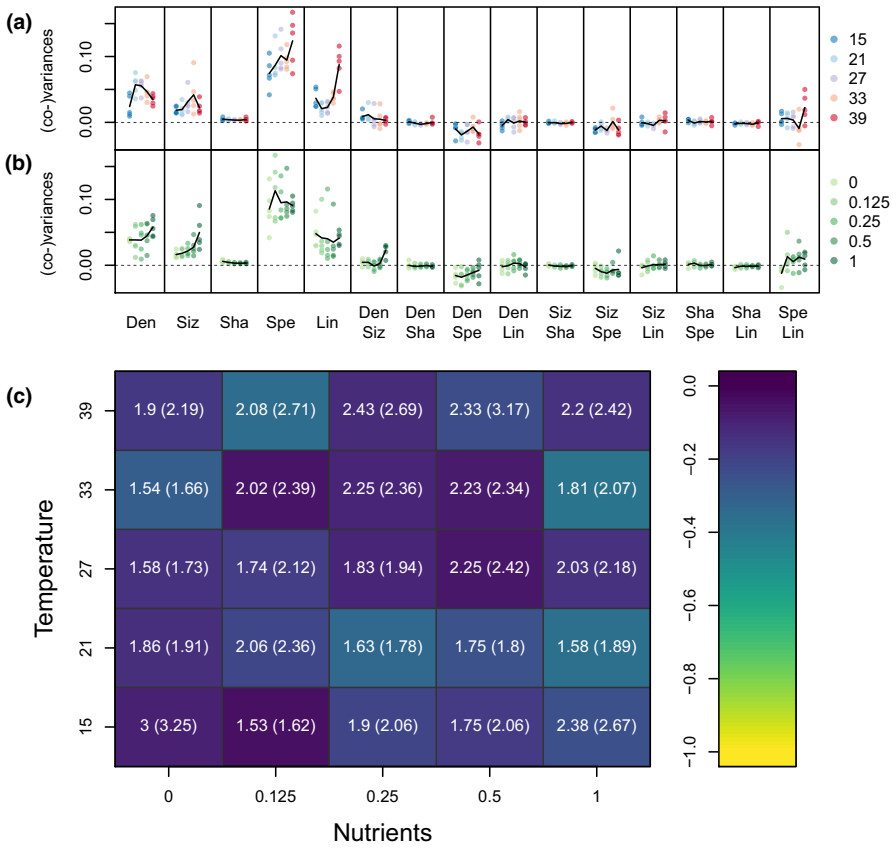
the variances was not as straightforward (Figure 4b). Overall, the covariances of the *G*-matrices were smaller in absolute values than the variances, and most of them changed sign depending on the environment considered. The effective number of dimensions of the *G*-matrices n_D ranged between 1.53 and 3 ($n_D = 1.98$ on average), indicating that the *G*-matrices were far from being full rank. However, this was also the case of the values of n_D', which ranged between 1.62 and 3.25 (Figure 4c). This suggests that the low dimensionality of the *G*-matrices was more likely due to heterogeneity in the variances of the five traits rather than to strong covariances among traits.

3.5 | Do genotypes differ in their plastic response to a given environmental gradient?

We found differences in raw trait expression between genotypes (*G* in Figure 2), with genotypes presenting consistently higher (or lower) trait values across the whole *T*, *N* or $T \times N$ environmental space (Figure 3). We also found a non-negligible variation among genotypes in their reaction norms to the two environmental factors ($G \times T + G \times N + G \times T \times N$ in Figure 2), which varied from 17.5% for size to 36.2% in movement speed. Response to temperature was more variable among genotypes than response to nutrient concentration ($G \times T \geq G \times N$ in Figure 2).

3.6 | Are some genotypes more plastic than others across all traits?

The rankings of the genotypes according to the coefficient of variation in their phenotype at each trait across each environmental gradient revealed substantial differences in the plasticity of the genotypes depending on the trait considered (Figure 5). Furthermore, some genotypes were significantly more plastic on average across all traits than others in their response to *T* (black dots in Figure 5 left column, Kruskall–Wallis $\chi^2_{df=14} = 27.388$, $p = 0.018$) but not in their response to *N* (black dots in Figure 5 right column, Kruskal–Wallis $\chi^2_{df=14} = 14.652$, $p = 0.402$). Interestingly, we did not find systematic differences in which genotypes were either highly plastic or highly canalized on average across all traits across the two environments. Genotypes that showed higher plasticity on average across all traits



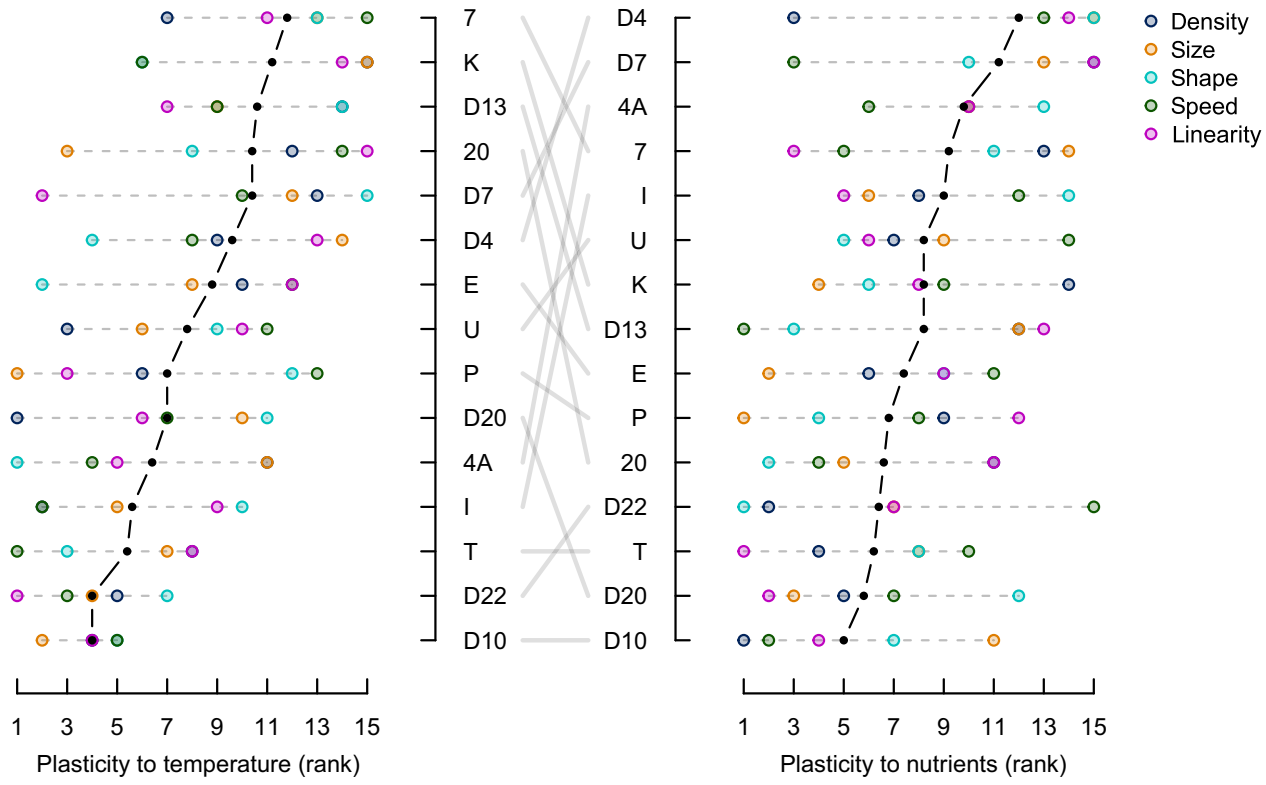


FIGURE 4 Values of variances and covariances of the five phenotypic traits (Den: density, Siz: size, Sha: shape, Spe: speed and Lin: linearity) for increasing values of temperatures (a) and for increasing values of nutrient concentration (b), with the average values (solid line). (c) Values of n_D (effective number of dimension) and n'_D (effective number of dimension with covariances forced at 0, in parentheses) for the 25 environmental combinations. The colours correspond to the values of r, expressing whether dimensionality reduction is due to heterogeneous variances ($r = 0$, purple) or to strong covariances ($r = 1$, yellow)

FIGURE 5 Plasticity magnitude rank of the genotypes (coefficient of variation of each trait across T and across N) for plasticity to temperature (left) and to nutrient concentration (right). The genotypes are sorted vertically according to their mean rank (black) across the five traits for each environmental factor. The rank positions of the genotypes in each gradient are connected by grey lines to compare average plasticity of genotypes across the two environmental gradients

for temperature were not those who were the most plastic on average across all traits for nutrient concentration (lines connecting genotypes between left and right panel in Figure 5).

4 | DISCUSSION

We experimentally quantified the plastic responses of five traits for 15 genotypes of *T. thermophila* along gradients of both temperature and nutrient concentration to answers six questions about correlational effects of phenotypic plasticity in systems with multiple environmental gradients. We showed that: (a) different environmental gradients had different effects on a given trait, (b) temperature and nutrient concentrations generated additive impacts on the plastically induced phenotypes, (c) a number of traits showed similar reaction norms to a given environmental gradient, but neither gradient influenced all traits in the same way, (d) no strong covariance between traits was conserved across environmental gradients, (e) the genotypes differed in their plastic response to a given environmental gradient and (f) the genotypes differed in their overall plasticity across all traits. Our experiment led us to explore different axes of multidimensionality of the phenotypic response: the inter-environment axis by testing all combinations of five values for both environmental gradients, the inter-trait axis by measuring five different phenotypic traits, and the inter-genotype axis by using 15 genetically distinct genotypes.

4.1 | The inter-environment axis

We investigated plastic responses of *T. thermophila* to variation in nutrient concentration and temperature to ask how different environments influence specific traits. Interestingly, only a single trait—final density, a measure of demographic population growth—actually responded to both environmental gradients. Such a plastic response to multiple environmental factors could result from the response of organisms to a single cue, itself correlated with multiple environmental gradients (Westneat, Hatch, Wetzel, & Ensminger, 2011; Westneat et al., 2019). For instance, the chrysophyte *Synura echinulate* responds in the same way to variations in light intensity and in temperature (Němcová, Neustupa, Kvíderová, & Řezáčová-Škaloudová, 2010), which are thought to both convey the same information about environmental stress triggering the plastic response. The plasticity in cell density observed in our experiment is unlikely to correspond to this case, given the differences in the shapes of the reaction norms to temperature and nutrient concentration. We observed two plastic responses of the same trait to different environmental gradients that likely influence the trait separately. This can notably occur for traits whose variation can be underpinned by multiple processes that can each be affected by the environment independently. For instance, the leaf mass per area (LMA) was found to be plastic to irradiance (Sack, Melcher, Liu, Middleton, & Pardee, 2006), nutrient abundance (Wright et al., 2005) and water abundance (Chin & Sillett, 2016), which actually all affect different aspects of the leaf structure, thereby changing the LMA value (Poorter, Niinemets, Poorter, Wright, & Villar, 2009). Similarly, the processes underpinning the cell division of *T. thermophila*, a component of fitness, depend on multiple factors related to the internal state of the cell and external cues. Therefore, density should exhibit plastic responses to multiple environmental gradients.

Although the plastic response to multiple environmental factors is seldom assessed, some studies have shown how interactions between multiple environmental gradients can affect the expression of reaction norms (Groot et al., 2016; Relyea, 2004; Stillwell et al., 2007), sometimes in a population-specific manner (Burghardt, 2016; Ris, Allemand, Fouillet, & Fleury, 2004; Stillwell et al., 2007). Here, the $T \times N$ interaction was systematically small in our experiment. Even the $G \times T \times N$ interaction remained comparatively low for every trait, ruling out the possibility that the absence of a $T \times N$ interaction was an artefact of opposite $T \times N$ effects among genotypes. Our results suggest that plasticity to temperature and nutrient concentration are additive, and therefore more easily predictable in regard to global environmental changes. However, the disparity of results across studies to date suggests that potential interactions between plastic responses to multiple environmental factors might depend on the trait, environmental factor and species considered. Further studies investigating the interaction between effects of multiple environments are necessary to see how general additivity of plastic responses to two or more environmental gradients is.

4.2 | The inter-trait axis

Understanding the relationship between the response of multiple traits to environmental gradients can shed light on past and future evolutionary changes of biological functions and especially multidimensional plasticity (Laughlin & Messier, 2015). Syndromes, that is, sets of covarying life-history traits, are found across taxa, for example, *r–K* strategies (Roff, 2002), pace-of-life (Réale et al., 2010), oogenesis-flight syndrome (Zera & Denno, 1997), pollination syndromes (Fenster, Armbruster, Wilson, Dudash, & Thomson, 2004) or dispersal syndromes (Legrand et al., 2016). Understanding these covariation patterns, their origins and how they are impacted by environmental conditions is critical to understand the evolvability of those functions but has rarely been examined for multiple plastic traits.

Despite some similarities in reaction norm shape to a given environmental gradient for different traits, the covariances among traits observed during the experiments were overall smaller than trait variances. Thus, we did not detect the dispersal syndrome, previously identified among *T. thermophila* (Fjerdingstad et al., 2007; Jacob, Chaine, Huet, Clobert, & Legrand, 2019; Jacob, Laurent, Morel-Journel, & Schtickzelle, 2019). Yet, our experimental design was designed to search for such a syndrome, as there

was no actual possibility for cells to disperse. However, the high variance of the traits could represent a strong potential for future evolution, especially for those traits linked to movement. The speed of individuals and the linearity of their trajectories differed greatly between genotypes, especially for the highest temperatures. More importantly, the covariances were generally inconsistent across environmental gradients. Such context dependency of trait covariation can have important ecological and evolutionary consequences. For instance, the lability of dispersal syndromes can allow organisms to adjust their dispersal movements according to both landscape characteristics and predatory risk (Winandy et al., 2019). Our study suggests that such labile trait covariation could be common, but we still lack empirical data to confirm this statement (but see Jacob, Laurent, et al., 2019; Legrand et al., 2016). Overall, the consistency of syndromes should be carefully assessed rather than assumed, especially in the most stressful conditions.

4.2.1 | The inter-genotype axis

An organism's genotype can influence its plastic response both through the value of its traits and through its plasticity. The raw values of the traits considered showed substantial variation linked to the genotype, as already shown in past studies (Fjerdingstad et al., 2007; Pennekamp et al., 2014), especially for traits linked to movement. Inter-genotype variation in traits linked to movement was especially high, and the lowest for cell density, which is consistent with standard selection models suggesting that traits more tightly linked to fitness are expected to show lower genetic variation (Kingsolver, Diamond, Siepielski, & Carlson, 2012; Palacio-López et al., 2015). The inter-genotypes differences in the plastic response underline the high potential for evolution of reaction norms among *T. thermophila*. Especially the partitioning of variances showed substantial $G \times E$ interactions, with $G \times T$ being systematically greater than $G \times N$, meaning that genotypes exhibited different reaction norms, especially to temperature. Those differences might reflect the environmental gradients encountered across the species' range, which spans from the north-eastern to the southern USA (Doerder, 2019; Zufall, Dimond, & Doerder, 2013). However, the latitudinal variation in the origin of the 15 genotypes used in this study is not big enough to allow formal testing for such an effect. Further studies linking the phylogeography of *T. thermophila* genotypes with the difference in their plastic response offer an opportunity to study the evolution of plasticity along natural environmental gradients.

Inter-genotype differences in plasticity at the trait level can translate into plasticity differences at the organism level (Forsman, 2015). In our experiment, we showed differences in overall plasticity of all five traits to temperature. These results are consistent with previous identification of thermal generalists and specialists among *T. thermophila* (Jacob et al., 2018), and with the differences in temperature variability across the specie's range (Doerder, 2019; Zufall et al., 2013). However, we did not find the same pattern in overall plasticity to nutrient concentration. Besides, there was no correlation between the overall plasticity to temperature and to nutrient concentration. In other words, the overall plasticity of one genotype to one environmental gradient did not predict its overall plasticity to another gradient, or even the existence of differences in overall plasticity between genotypes for that other gradient. Therefore, one should be cautious when separating specialists from generalists using a given environmental factor, as these definitions heavily depend on the environmental factor considered.

5 | CONCLUSIONS

This study addresses the multidimensional nature of phenotypic plasticity (inter-environmental, inter-trait and inter-genotype) in a single fully factorial experimental design. Our results have important implications for the evolution of phenotypic plasticity in nature where organisms are exposed to multiple environmental gradients simultaneously. We showed that plasticity in each trait depends on the environmental gradient, so does the existence of generalists and specialists, that is, genotypes that differed in their plasticity across all traits. At the trait level, the environmental effects on plasticity of a given trait are largely additive instead of having complex non-additive effects. Despite plasticity across multiple traits, there was little covariance among trait across environments, as would be expected if traits formed a stable life-history syndrome, most often as a consequence of genetic constraints. Together, these results suggest that plasticity of traits to different environments should evolve largely independently of other traits or each environmental cue rather than showing complex correlational evolutionary responses. However, this also means findings concerning plasticity of one trait to one environmental gradient are more difficult to generalize to other traits or environments, even for the same organism.

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AUTHORS' CONTRIBUTIONS

A.S.C., V.T. and N.S. conceived and designed research questions and the methodology for the experiment; V.T., F.P. and E.L. collected the data, using the digital image analysis workflow developed by F.P. and N.S.; T.M.-J., V.T. and N.S. analysed the data with contributions from D.L. and A.S.C.; T.M.-J. led the writing on the manuscript, with substantial contributions from A.S.C., D.L. and N.S. All the authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.612jm641r (Morel-Journel et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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