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1 **Title:** A multidimensional approach to the expression of phenotypic plasticity

2

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33

34 **Author Contributions**

35 ASC, VT and NS conceived and designed research questions and the methodology for the experiment.
36 VT, FP and EL collected the data, using the digital image analysis workflow developed by FP and NS.
37 TMJ, VT and NS analysed the data with contributions from DL and ASC. TMJ led the writing on the
38 manuscript, with substantial contributions from ASC, DL and NS. All authors contributed critically to
39 the drafts and gave final approval for publication.

40

41 **Data Availability Statement**

42 Should the manuscript be accepted, the experimental data supporting the results will be archived in
43 Dryad and the data DOI will be included at the end of the article.

44

45 **Abstract**

46 Phenotypic plasticity is increasingly recognized as a key element of eco-evolutionary dynamics, but it
47 remains challenging to assess because of its multidimensional nature. Indeed, organisms live in
48 complex environments where numerous factors can impact the phenotypic expression of traits
49 (inter-environment axis), possess multiple traits that can influence each other's expression (inter-
50 trait axis), and differ in their genetic background (inter-genotype axis), which can not only impact the
51 traits' values but also their plasticity. We designed a microcosm experiment using the protist
52 *Tetrahymena thermophila* aimed at encompassing all these aspects of phenotypic plasticity. We
53 exposed 15 distinct genotypes to 25 combinations of temperature and nutrient availability and
54 assessed the plasticity of five phenotypic traits. Hence, we addressed six questions related to
55 phenotypic plasticity: (1) do different environmental gradients show similar effects on a given trait?
56 (2) Are the effects of two environmental gradients on a trait additive? (3) Do different traits show
57 similar plastic response to a given environmental gradient? (4) Do the (co)variances between traits
58 vary across environmental gradients? (5) Do genotypes differ in their plastic response to a given
59 environmental gradient? (6) Are some genotypes more plastic than others across all traits? Our
60 results show strong differences in the plastic response depending on the environmental gradient, not
61 only regarding the shape of the reaction norm of the different traits tested, but also in the overall
62 plasticity of the organisms. We did not find any covariance between traits that was consistent across
63 all environments. Overall, our results suggest independent impacts of the environmental dimension
64 considered on the observed plastic response. These results underline potential difficulties in
65 generalising findings about plasticity to all environments and all traits.

66 **Keywords (8 words)**

67 controlled microcosms, G-matrix, genotype x environment experiment, phenotypic plasticity,

68 phenotypic syndrome, *Tetrahymena thermophila*, variance partitioning

69

70 **Introduction**

71 Phenotypic plasticity is increasingly recognized as a critical factor in mediating eco-evolutionary
72 processes (Carroll, Hendry, Reznick, & Fox, 2007; DeWitt & Scheiner, 2004; Forsman, 2015; Hendry,
73 2015; Pigliucci, 2001). Research over the past decades has revealed the ubiquity of plasticity (Miner,
74 Sultan, Morgan, Padilla, & Relyea, 2005; Palacio-López, Beckage, Scheiner, & Molofsky, 2015;
75 Pigliucci, 2001; Price, Qvarnström, & Irwin, 2003; West-Eberhard, 2003) and identified the
76 developmental (Emlen & Nijhout, 2000; Hoverman & Relyea, 2007; Murren et al., 2015), genetic
77 (Callahan, Dhanoolal, & Ungerer, 2005; DeWitt & Scheiner, 2004; Van Kleunen & Fischer, 2005), and
78 epigenetic (Auge, Leverett, Edwards, & Donohue, 2017; Ledón-Rettig, Richards, & Martin, 2012;
79 Smith & Ritchie, 2013) processes underlying plasticity. Plasticity occurs when a given genotype
80 produces different phenotypes in response to external factors, which may increase its short-term
81 success when confronted with varying environments (DeWitt & Scheiner, 2004; Pigliucci, 2001). The
82 value of such plasticity ranges from buffering the impacts of changing environmental conditions
83 (Ghalambor, McKay, Carroll, & Reznick, 2007; Reed, Waples, Schindler, Hard, & Kinnison, 2010) to
84 facilitating colonization of new habitats (Geng et al., 2016; Richards, Bossdorf, Muth, Gurevitch, &
85 Pigliucci, 2006; Yeh & Price, 2004), and helping to cross “adaptive valleys” during population
86 divergence and speciation (Agrawal, 2001; Pfennig et al., 2010; van Snick Gray & Stauffer, 2004).

87 Much of the early research on phenotypic plasticity has focused on the response of traits to a
88 single environmental gradient (e.g. Bruno & Edmunds, 1997; Denver, Mirhadi, & Phillips, 1998;
89 Weider & Pijanowska, 1993). Yet, natural environments are composed of a multitude of interacting
90 factors potentially influencing the plastic expression of traits. For example, the growth rate of the
91 seed beetle *Callosobruchus maculatus* varies with both temperature and the rearing host (Stillwell,
92 Wallin, Hitchcock, & Fox, 2007), and the laying date of great tit *Parus major* varies with both
93 temperature and day-length (Gienapp, Väisänen, & Brommer, 2010). However, our understanding of
94 how multiple environmental gradients influence reaction norms remains limited (Westneat, Potts,
95 Sasser, & Shaffer, 2019). The effect of two environmental dimensions could be additive, antagonistic,

96 synergistic or complementary. Determining how multiple environments influence plasticity is
97 challenging because it requires measures of independent and joint effects of factors on the same
98 genotype through fully factorial experimental designs.

99 Studies concerning multivariate plasticity focus on the interaction between multiple
100 phenotypic traits and how each trait affects phenotypic plasticity in the other trait (Laughlin &
101 Messier, 2015; Westneat et al., 2019). Indeed, organisms are mosaics of traits interacting to
102 influence fitness (Dochtermann & Dingemanse, 2013; Legrand et al., 2016; Sih, Bell, & Johnson,
103 2004), which raises several questions. Firstly, will a single environmental factor cause plastic changes
104 on several traits, or will traits be independently influenced by different factors? Secondly, for a given
105 environmental factor, will the change in each trait be in a similar direction and magnitude, and how
106 will the covariances between traits be impacted? While considerable information has accumulated
107 on the shapes of reaction norms for a single trait measured along a single environmental gradient,
108 we still need to determine if reaction norms are generalizable across traits and/or environmental
109 factors.

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

118 Phenotypic plasticity is therefore a complex phenomenon involving different kinds of multi-
119 dimensionalities, usually addressed within separate studies. We identified three different axes along
120 which this multidimensionality deserves deeper investigation (Fig. 1): (i) the inter-environment axis,
121 concerning interactions between the response to multiple environmental factors (Westneat et al.,

122 2019), (ii) the inter-trait axis, concerning the interactions between the responses of multiple traits to
123 the same environmental factors (Laughlin & Messier, 2015), and (iii) the inter-genotype axis,
124 concerning the relationship between the genetic background of organisms and their plastic response
125 to environmental factors (Pigliucci, Murren, & Schlichting, 2006; Richards et al., 2006).

126 In this study, we investigated the complex interplay between these three axes in the
127 expression of phenotypic plasticity. We used a series of controlled fully-factorial microcosm
128 experiments in 15 clonal genotypes of a ciliated protist (*Tetrahymena thermophila*), where we
129 manipulated temperature and nutrient concentration and measured the expression of five
130 phenotypic traits linked to fitness, morphology and movement. These two environmental factors
131 were chosen as they are both likely to vary over space and time in the natural habitat (freshwater
132 bodies), where *T. thermophila* feeds on bacteria. Furthermore, with increasing temperature,
133 metabolism accelerates leading to higher resource demand (Gerhard, 2019). This effect can be
134 compensated by higher nutrient supply. The existence of phenotypic plasticity and its variation
135 among genotypes have been shown previously in this species for various morphological, behavioural
136 and fitness traits, but only along single environmental gradients, e.g., density-dependence of
137 dispersal (Pennekamp, Mitchell, Chaine, & Schtickzelle, 2014) and thermal performance (Jacob et al.,
138 2018). Here, we examined plasticity in morphological, behavioural and fitness traits along two
139 different environmental gradients, for each genetic line. We aimed at answering six questions (Fig.
140 1): (1) Do different environmental gradients show similar effects on a given trait? (2) Are the effects
141 of two environmental gradients on a trait additive? (3) Do different traits show similar plastic
142 responses to a given environmental gradient? (4) Do the (co)variances between traits vary across
143 environmental gradients? (5) Do genotypes differ in their plastic response to a given environmental
144 gradient? (6) Are some genotypes more plastic than others across all traits?

145 **Material and Methods**

146 *Strains and cultures*

147 We used 15 isogenic lines (referred to as “genotypes”) of *Tetrahymena thermophila*, a free-living
148 unicellular eukaryote (Supporting table 1). This species has been used for decades as a model species
149 in molecular biology and physiology and more recently in evolutionary ecology (Chaine, Schtickzelle,
150 Polard, Huet, & Clobert, 2010; Fjerdingstad, Schtickzelle, Manhes, Gutierrez, & Clobert, 2007; Jacob,
151 Clobert, Legrand, Schtickzelle, & Chaine, 2016; Jacob et al., 2018, 2017; Pennekamp et al., 2014;
152 Schtickzelle, Fjerdingstad, Chaine, & Clobert, 2009). The genotypes used for this study were either
153 isolated from natural populations across different sites in North America or created by subsequent
154 crossings between genotypes in the laboratory (Supporting table 1). They were cultivated in light-
155 controlled incubators (14h light/10h dark-cycle at 27°C) in an axenic medium (PPYE 1x: 2% Proteose
156 Peptone and 0.2% Yeast Extract [Becton Dickinson] diluted in ultrapure water [Altermatt et al.,
157 2015]). Culture stocks were renewed every 10 days by inoculating a 2ml sample of fresh medium
158 with 100µl of culture and maintained in 2ml 24-well plates (CELLSTAR ref. 662160, Greiner BioOne,
159 Belgium). All manipulations of axenic cultures were conducted under sterile conditions in a laminar
160 flow hood (Ultrasafe 218 S, Faster, Italy).

161

162 *Experimental design*

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

171 x 5 N). The whole experiment was replicated three times leading to three fully independent
172 replicates for each combination; sample size for analysis then equals to 1125 “G×N×T×replicate”
173 cultures.

174 Before each of the three repetitions of the experiment, one mother culture was initiated per
175 genotype, by inoculating 5ml of PPYE 1x with 500µl of monoculture of a given genotype and
176 incubated in the standard culture conditions (27°C, PPYE 1x) for two days. Each of these 15 “G”
177 mother cultures was then transferred in 100ml of fresh PPYE 1x, conserved for five additional days in
178 the same conditions to reach sufficient cell concentration for the experiment. Then, they were
179 carefully homogenized and aliquoted into five smaller tubes, from which the old medium was
180 removed by aspiration after five minutes of centrifugation at 250g and replaced by 10ml of fresh
181 PPYE nutrient, for each of the five different nutrient concentration levels. Finally, each of these 75
182 “G×N” culture was diluted to a standardized density of 300 kcell/ml with fresh PPYE medium at the
183 same concentration, split into five 4ml cultures, each incubated at one of the five temperatures
184 tested to start the experiment, giving 375 “G×N×T” cultures. Data were collected at two times during
185 the experiment on these 375 “G×N×T” cultures. The cell density was measured after 24 hours, as a
186 proxy for demography (population growth). Then, each culture was standardized at a density of 100
187 kcell/ml through dilution into fresh medium and re-incubated with their respective nutrient
188 concentration and temperature for one additional hour. The main reason to proceed in this way was
189 to avoid a potential bias in the measure of movement behaviour arising when cell density is too high.
190 In such conditions, “collisions” between swimming cells blur cell identity during tracking, cutting each
191 movement trajectory into shorter and straighter bits, biasing speed and linearity. On the contrary,
192 morphology is not largely affected over such a short time (1h) given that temperature and nutrient
193 concentration conditions were unchanged.

194 Five metrics related to morphology, behaviour and fitness were quantified during the
195 experiment using our standardized digital picture workflow (Pennekamp et al., 2014; Pennekamp,
196 Schtickzelle, & Petchey, 2015; Pennekamp & Schtickzelle, 2013): density, cell size, cell shape,

197 movement speed and movement linearity. For each culture, five 10 μ l samples were extracted after
198 culture homogenization and each loaded into a chamber of a counting slide (Precision cell, Kima,
199 Italy). The first three variables were obtained by analysing the pictures taken of each chamber under
200 a dark field microscope: the density was estimated from the number of cells counted after 24 hours,
201 the cell size as the area of the cross section in μm^2 , and the cell shape as the aspect ratio (*i.e.*,
202 major/minor axis ratio of a fitted ellipse; the minimal shape value was 1 for a perfectly round cell).
203 The last two variables concerning movement were obtained from one video taken for a randomly
204 chosen chamber among the five available for each culture. The videos were analysed using the
205 BEMOVI R package (Pennekamp et al., 2015; <http://www.bemovi.info>) to reconstruct movement
206 trajectories and obtain movement speed and linearity; linearity was quantified as the net-to-gross
207 distance ratio (net distance is the Euclidian distance between the starting and arrival points; gross
208 distance is the effective length of the trajectory; the maximal linearity value of 1 then indicates a
209 perfectly straight path).

210

211 *Analysis of the results*

212 The statistical analyses of the experimental results were all performed using the R software (R Core
213 team, 2018). To answer questions 1, 2, 3 and 5, the response variables (*i.e.*, the five phenotypic
214 traits) were analysed using an ANOVA for variance partitioning and a random regression-mixed
215 model for describing the mean reaction norm. First, we used an ANOVA model to quantify how the
216 variance in the trait values is explained by the temperature (T), the nutrient concentration (N), the
217 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
218 the number of levels of each environmental factor (5) and of genotypes (15), we performed the
219 variance partitioning for every possible subset of 5 genotypes among the 15. For each term, we
220 estimated the respective effect size of each factor and their interaction using the η^2 metric (Fritz,
221 Morris, & Richler, 2012), and then we computed the average value of η^2 over all these subsets as
222 metrics of trait variance.

223 Reaction norms, *i.e.*, average trait values over the two-dimensional space of temperature and
224 nutrient concentration, were assessed using quadratic random regression-mixed model analyses
225 (Morrissey and Liefting 2016) using the *lme4* package (Bates et al., 2014). For each trait, the mixed
226 model took the following form:

$$227 \quad z_{i,G} = a + b_1T_i + b_2T_i^2 + b_3N_i + b_4N_i^2 + b_5T_iN_i + c + d_{1,G}T_i + d_{2,G}T_i^2 + d_{3,G}N_i + d_{4,G}N_i^2 + d_{5,G}T_iN_i + e_i$$

228 with $z_{i,G}$ the value of the trait for the experimental replicate i for the genotype g exposed to a given
229 temperature T_i and a given nutrient concentration N_i . To limit the size of the covariance matrix to
230 ensure estimation of random effects and convergence of the models, we limited the interactions to
231 the linear terms (T_i and N_i). Indeed, the inability to include interaction terms in the random slopes
232 model is why we partitioned variance using an ANOVA approach above. The coefficients a , b_1 , b_2 , b_3 ,
233 b_4 and b_5 were used to estimate the reaction norm, while accounted for potential non-linear
234 relationships between the trait values and the environment, as well as the interactions between the
235 environmental gradients. T and N values were each centred and standardized prior to the analysis to
236 ensure independence between linear and quadratic terms as well as comparability of their size effect
237 despite being expressed in different units (Shielzeth, 2010).

238 To answer question 4, for every environmental condition (the 5x5 environmental
239 combinations), a G-matrix was computed to describe the (co)variances between the values of each
240 trait across all genotypes. Each G-matrix included the variance of each trait on its diagonal, and the
241 covariances between the five traits off diagonal. Since G-matrices are symmetrical, the covariances in
242 the upper and lower triangles were identical and each matrix included 15 unique values: 5 variances
243 and 10 covariances. To account for differences in the ranges of the trait values (*e.g.*, linearity is a
244 ratio always between 0 and 1 while density is always a positive integer), a mean standardization was
245 performed by dividing the raw values by the mean value for each environmental condition (Delahaie
246 et al., 2017; Kirkpatrick, 2009). We computed the effective number of dimensions of each G-matrix
247 n_D , which can theoretically vary between 1 (the G-matrix is singular) to the number of traits (the G-
248 matrix is full-rank; here 5). The value of n_D decreases when fewer dimensions are enough to describe

249 the full information content in the G-matrix; and this can happen because of existing covariation
250 between traits (one trait explains variation in other traits) and/or heterogeneity in the level of
251 variance in the different traits (if one trait has a much higher variance than others, it alone explains a
252 high proportion of the matrix information, hence decreasing n_D) (Kirkpatrick, 2009). To disentangle
253 the relative importance of these two aspects in giving low n_D values, we computed an extra measure
254 n_D' , corresponding to n_D obtained in a set of 25 G-matrices with variances identical to the observed
255 ones but covariances forced to 0; this n_D' value sets the maximum number of effective dimensions in
256 the absence of covariance between traits but taking heterogeneous trait variances into account.
257 Then, we computed the ratio $((n_D - 1) - (n_D' - 1)) / (n_D' - 1)$ to quantify the reduction in effective
258 number of dimensions in the G-matrices due to the existence of covariances among traits.

259 Finally, to answer question 6, the coefficient of variation of each trait was computed for each
260 genotype across T and across N as a proxy for the magnitude of their plasticity. The genotypes were
261 ranked according to these coefficients (from 1 for the least plastic to 15 for the most plastic), and
262 these ranks were compared using Kruskal-Wallis tests to identify differences in the average plasticity
263 of genotypes over the five measured traits.

264

265 **Results**

266 *(1) Do different environmental gradients show similar effects on a given trait?*

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

275

276 *(2) Are the effects of two environmental gradients on a trait additive?*

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

281

282 *(3) Do different traits show similar plastic responses to a given environmental gradient?*

283 While some traits showed similar plastic responses to a given environmental gradient, neither
284 manipulated environmental feature influenced all traits in the same way. Density, cell shape and
285 movement were consistently influenced by T. Interestingly, reaction norms of these traits were all
286 quadratic (Fig. 3) and all reached their inflection points (maximum density, maximally elongated
287 shape and minimal movement linearity) for intermediate values of T. Density and cell size were
288 consistently plastic to N. They exhibited a strong reduction of trait values at the lowest concentration
289 (0x) compared to the other ones.

290

291 *(4) Do the (co)variances between traits vary across environmental gradients?*

292 There were large differences between the variances of the different traits, for all the G-matrices (Fig.
293 4A, 4B). The variance in shape was systematically low for all environments, while variances in speed
294 and in linearity were on average higher, and tended to increase with temperature (Fig 4A). The
295 impact of nutrient concentration on the variances was not as straightforward (Fig 4B). Overall, the
296 co-variances of the G-matrices were smaller in absolute values than the variances, and most of them
297 changed sign depending on the environment considered. The effective number of dimensions of the
298 G-matrices n_D ranged between 1.53 and 3 ($n_D = 1.98$ on average), indicating that the G-matrices were
299 far from being full rank (Fig 4C). However, this was also the case of the values of n_D' , which ranged
300 between 1.62 and 3.25. This suggests that the low dimensionality of the G-matrices is more likely due

301 to heterogeneity in the variances of the five traits rather than to strong covariances among traits in
302 the G-matrices.

303

304 *(5) Do genotypes differ in their plastic response to a given environmental gradient?*

305 We found considerable differences in raw trait expression between genotypes (G in Fig. 2), with
306 genotypes presenting consistently higher (or lower) trait values across the whole T, N or T×N
307 environmental space (Fig. 3). We also found a non-negligible variation among genotypes in their
308 reaction norms to the two environmental factors (G×T + G×N + G×T×N in Fig. 2), which varies from
309 17.5% for size to 36.2% in movement speed. Response to temperature was more variable among
310 genotypes than response to nutrient concentration (G×T ≥ G×N in Fig. 2).

311

312 *(6) Are some genotypes more plastic than others across all traits?*

313 The rankings of the genotypes according to the coefficient of variation in their phenotype at each
314 trait across each environmental gradient revealed substantial differences in the plasticity of the
315 genotypes depending on the trait considered (Fig. 5). Furthermore, some genotypes were
316 significantly more plastic on average across all traits than others in their response to T (black dots in
317 Fig. 5 left column; Kruskal-Wallis $\chi^2_{df=14} = 27.388$; $p = 0.018$) but not in their response to N (black
318 dots in Fig. 5 right column; Kruskal-Wallis $\chi^2_{df=14} = 19.442$; $p = 0.149$). Interestingly, we did not find
319 systematic differences in which genotypes were either highly plastic or highly canalized on average
320 across all traits across the two environments. Genotypes that showed higher plasticity on average
321 across all traits for temperature were not those who were the most plastic on average across all
322 traits for nutrient concentration (lines connecting genotypes between left and right panel in Fig. 5).

323

324 **Discussion**

325 We experimentally quantified the plastic responses of five traits for 15 genotypes of *T. thermophila*
326 along gradients of both temperature and nutrient concentration to answer six questions about

327 correlational effects of phenotypic plasticity in systems with multiple environmental gradients. We
328 showed that: (1) different environmental gradients had different effects on a given trait, (2)
329 temperature and nutrient concentrations generated additive impacts on the plastically-induced
330 phenotypes, (3) a number of traits showed similar reaction norms to a given environmental gradient,
331 but neither gradient influenced all traits in the same way, (4) no strong covariance between traits
332 was conserved across environmental gradients, (5) the genotypes differed in their plastic response to
333 a given environmental gradient, and (6) the genotypes differed in their overall plasticity across all
334 traits. Our experiment led us to explore different axes of multidimensionality of the phenotypic
335 response: the inter-environment axis by testing all combinations of five values for both
336 environmental gradients, the inter-trait axis by measuring five different phenotypic traits, and the
337 inter-genotype axis by using fifteen genetically distinct genotypes.

338

339 *The inter-environment axis*

340 We investigated plastic responses of *T. thermophila* to variation in nutrient concentration and
341 temperature to ask how different environments influence specific traits. Interestingly, only a single
342 trait –final density, a measure of demographic population growth– responded extensively to both
343 environmental gradients. Such a strong plastic response to multiple environmental factors could
344 result from the response of organisms to a single cue, itself correlated with multiple environmental
345 gradients (Westneat, Hatch, Wetzel, & Ensminger, 2011; Westneat et al., 2019). For example, the
346 chrysophyte *Synura echinulate* responds in the same way to variations in light intensity and in
347 temperature (Němcová, Neustupa, Kvíderová, & Řezáčová-Škaloudová, 2010), which are thought to
348 both convey the same information about environmental stress triggering the plastic response. The
349 plasticity in cell density observed in our experiment is unlikely to correspond to this case, given the
350 differences in the shapes of the reaction norms to temperature and nutrient concentration. We
351 observed two plastic responses of the same trait to different environmental gradients that likely
352 influence the trait separately. This can notably occur for traits whose variation can be underpinned

353 by multiple processes that can each be affected by the environment independently. For instance, the
354 leaf mass per area (LMA) was found to be plastic to irradiance (Sack, Melcher, Liu, Middleton, &
355 Pardee, 2006), nutrient abundance (Wright et al., 2005) and water abundance (Chin & Sillett, 2016),
356 which actually all affect different aspects of the leaf structure, thereby changing the LMA value
357 (Poorter, Niinemets, Poorter, Wright, & Villar, 2009). Similarly, the processes underpinning the cell
358 division of *T. thermophila*, a component of fitness, depend on multiple factors related to the internal
359 state of the cell and external cues. Therefore, density should exhibit plastic responses to multiple
360 environmental gradients.

361 Although the plastic response to multiple environmental factors is seldom assessed, some
362 studies have shown how interactions between multiple environmental gradients can affect the
363 expression of reaction norms (Groot et al., 2016; Relyea, 2004; Stillwell et al., 2007), sometimes in a
364 population-specific manner (Burghardt, 2016; Ris, Allemand, Fouillet, & Fleury, 2004; Stillwell et al.,
365 2007). Here, the T×N interaction was systematically negligible in our experiment. Even the G×T×N
366 interaction remained comparatively low for every trait, ruling out the possibility that the absence of a
367 T×N interaction was an artefact of opposite T×N effects among genotypes. Our results suggest that
368 plasticity to temperature and nutrient concentration are additive, and therefore more easily
369 predictable in regard to global environmental changes. However, the disparity of results across
370 studies to date suggests that potential interactions between plastic responses to multiple
371 environmental factors might depend on the trait, environmental factor and species considered.
372 Further studies investigating the interaction between effects of multiple environments are necessary
373 to see how general additivity of plastic responses to two or more environmental gradients is.

374

375 *The inter-trait axis*

376 Understanding the relationship between the response of multiple traits to environmental gradients
377 can shed light on past and future evolutionary changes of biological functions and especially multi-
378 dimensional plasticity (Laughlin & Messier, 2015). Syndromes, *i.e.*, sets of covarying life-history traits,

379 are found across taxa, *e.g.*, r-K strategies (Roff, 2002), pace-of-life (Réale et al., 2010), oogenesis-
380 flight syndrome (Zera & Denno, 1997), pollination syndromes (Fenster, Armbruster, Wilson, Dudash,
381 & Thomson, 2004) or dispersal syndromes (Legrand et al., 2016). Understanding these covariation
382 patterns, their origins and how they are impacted by environmental conditions is critical to
383 understand the evolvability of those functions but has rarely been examined for multiple plastic
384 traits.

385 Despite some similarities in reaction norm shape to a given environmental gradient for
386 different traits, the covariances among traits observed during the experiments were actually largely
387 smaller than trait variances. Indeed, the values on the diagonal of the G-matrices were overall
388 greater than off diagonal values. Thus, we did not find the dispersal syndrome, previously identified
389 among *T. thermophila* (Fjerdingstad et al., 2007; Jacob, Chaine, Huet, Clobert, & Legrand, 2019;
390 Jacob, Laurent, Morel-Journel, & Schtickzelle, 2019); however our experimental design was not
391 suitable to search for such a syndrome, as there was no possibility for cells to disperse. However, the
392 high variance of the traits could represent a strong potential for future evolution, especially for those
393 traits linked to movement. The speed of individuals and the linearity of their trajectories differed
394 greatly between genotypes, especially for the highest temperatures. More importantly, the
395 covariances were inconsistent across environmental gradients. Such context-dependency of trait
396 covariation can have important ecological and evolutionary consequences. For instance, the lability
397 of dispersal syndromes can allow organisms to adjust their dispersal movements according to both
398 landscape characteristics and predatory risk (Winandy et al., 2019). Our study suggests that such
399 labile trait covariation could be common, but we still lack empirical data to confirm this statement
400 (but see Jacob, Laurent, et al., 2019; Legrand et al., 2016). Overall, the consistency of syndromes
401 should be carefully assessed rather than assumed, especially in the most stressful conditions.

402

403 *The inter-genotype axis*

404 An organism's genotype can influence its plastic response both through the value of its traits and

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

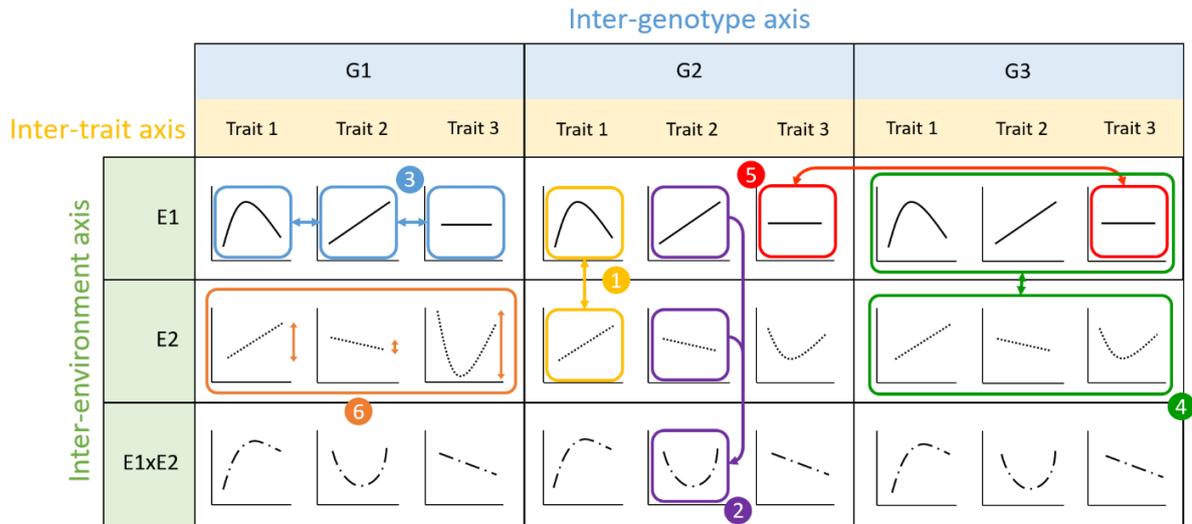
421 Inter-genotype differences in plasticity at the trait level can translate into plasticity
422 differences at the organism level (Forsman, 2015). In our experiment, we showed differences in
423 overall plasticity of all five traits to temperature. These results are consistent with previous
424 identification of thermal generalists and specialists among *T. thermophila* (Jacob et al., 2018), and
425 with the differences in temperature variability across the specie's range (Doerder, 2019; Zufall et al.,
426 2013). However, we did not find the same pattern in overall plasticity to nutrient concentration.
427 Indeed, we did not find genotypes that were highly plastic overall, or plasticity specialists relative to
428 others. Moreover, there was no correlation between the overall plasticity to temperature and to
429 nutrient concentration. In other words, the overall plasticity of one genotype to one environmental
430 gradient did not predict its overall plasticity to another gradient, or even the existence of differences

431 in overall plasticity between genotypes for that other gradient. Therefore, one should be cautious
432 when separating specialists from generalists using a given environmental factor, as these definitions
433 heavily depend on the environmental factor considered.

434 **Conclusions**

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

449

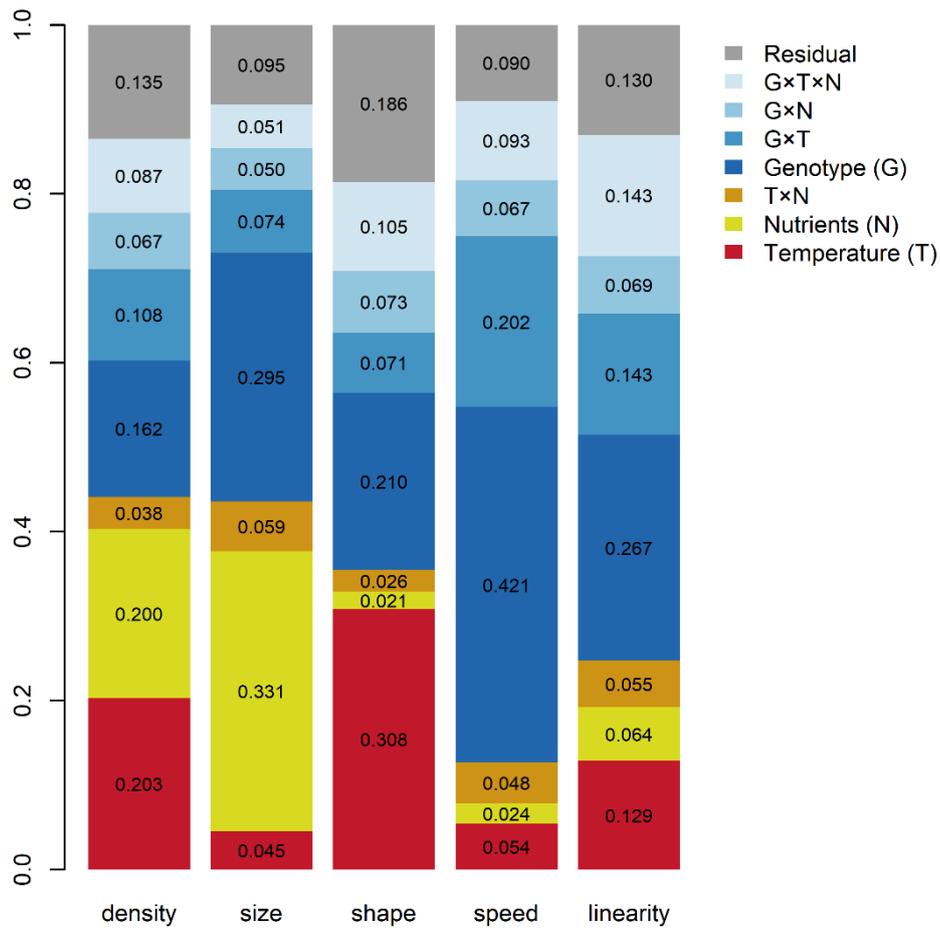


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451

452 **Figure 1:** Schematic representation of the six different questions considered in this study and their
 453 relationship to the three identified multidimensionality axes. We have represented hypothetical
 454 reaction norms of three traits for three genotypes (G1, G2, G3) in two environmental gradients (E1,
 455 E2) and their interaction (E1x2). We asked: (1) if different environmental gradients show similar
 456 effects on the plasticity of a given trait, (2) if the effects of two environmental gradients on a trait are
 457 additive, (3) if different traits show similar plastic response to a given environmental gradient, (4) if
 458 (co)variances between traits vary across environmental gradients, (5) if there are differences
 459 between genotypes in their plastic response to a given environmental gradient, and (6) if some
 460 genotypes are more plastic than others across all traits. Each question is illustrated with a single
 461 example comparison only.

462

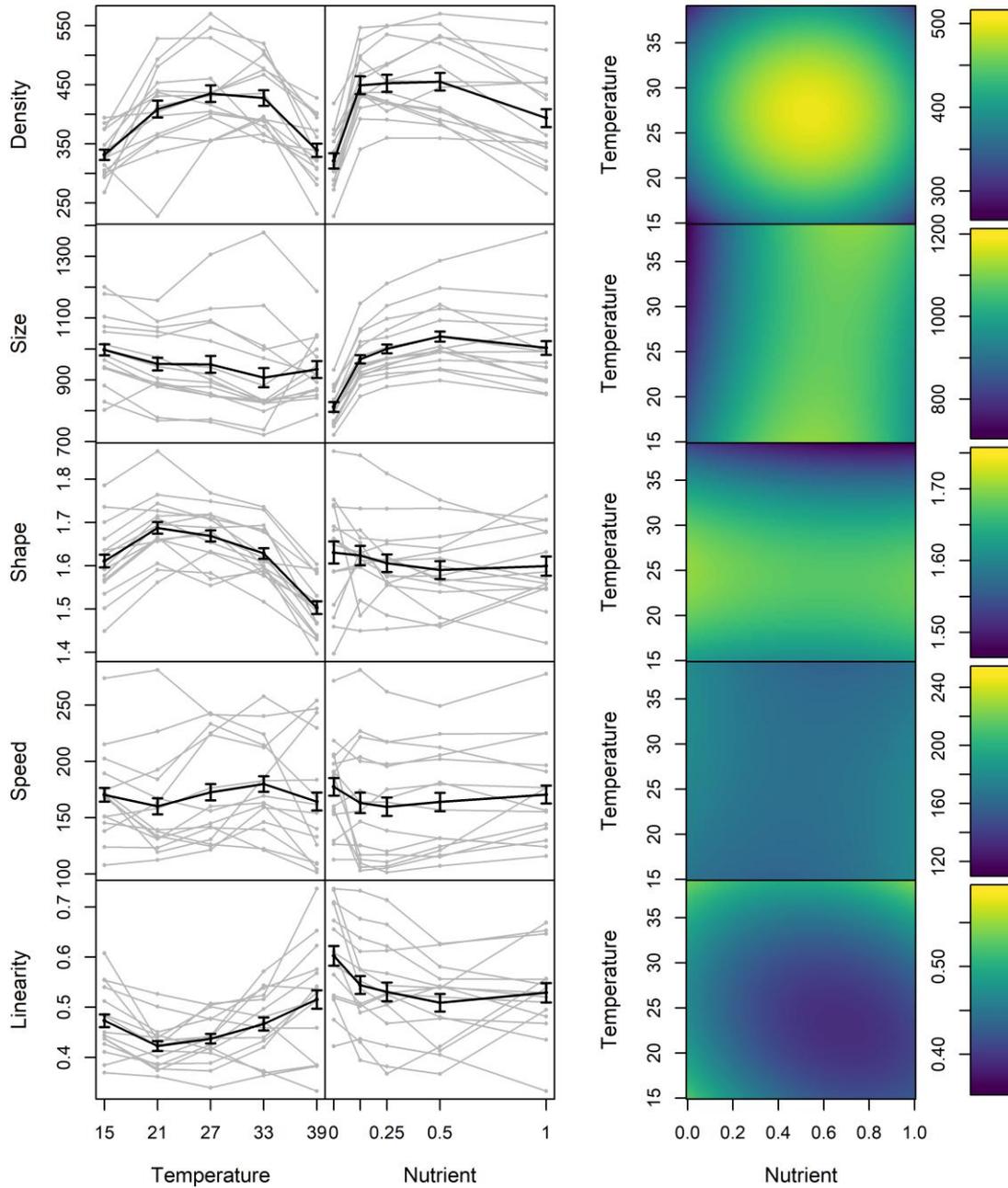


463

464

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

469



470

471

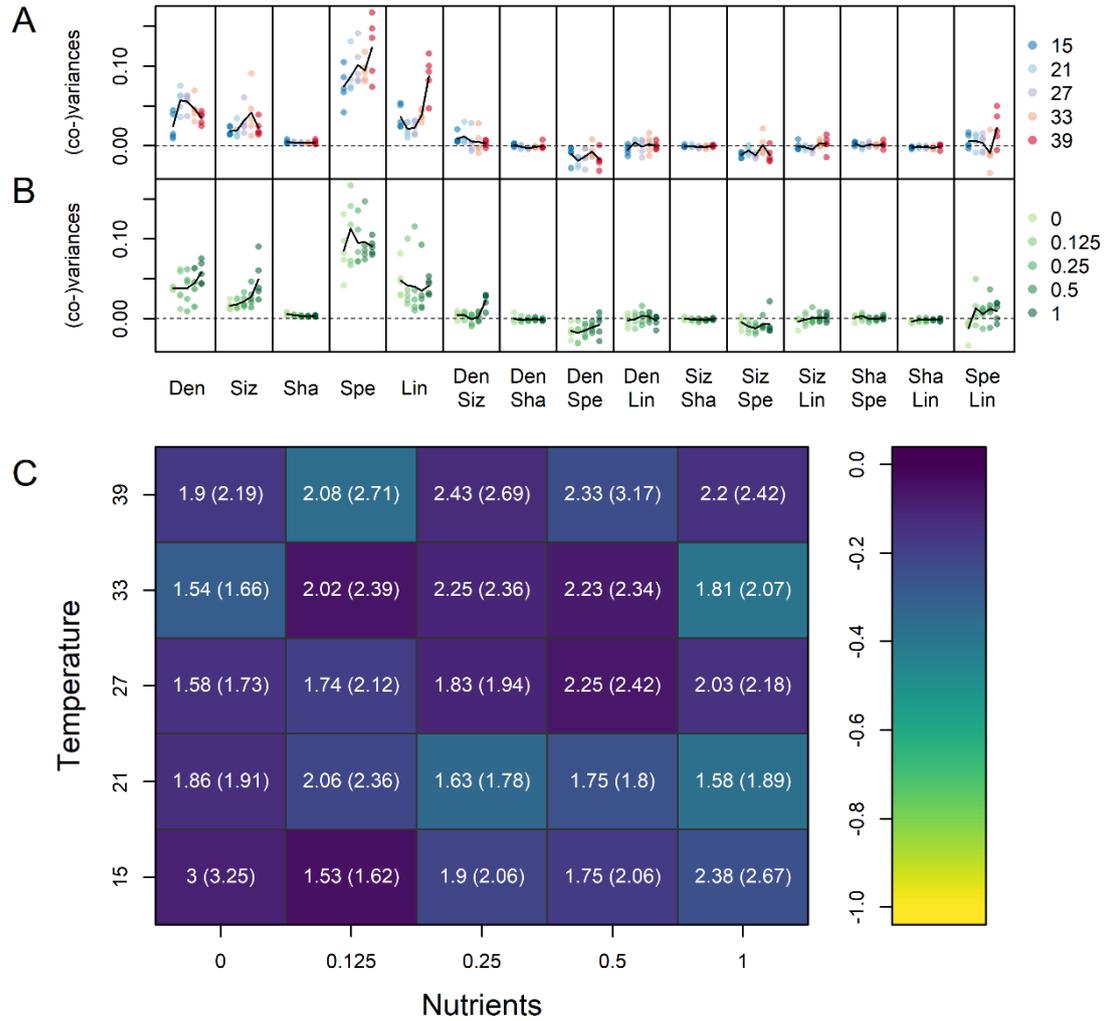
472 **Figure 3:** Reaction norms of the five traits according to the two environmental gradients

473 (temperature and nutrient concentration). **(Left)** Observed values for each genotype (grey lines,

474 averaged over 3 replicates) and averaged over all genotypes (black line \pm 2 standard errors) for each

475 environmental gradient separately. **(Right)** Predicted mean reaction norms across the T x N

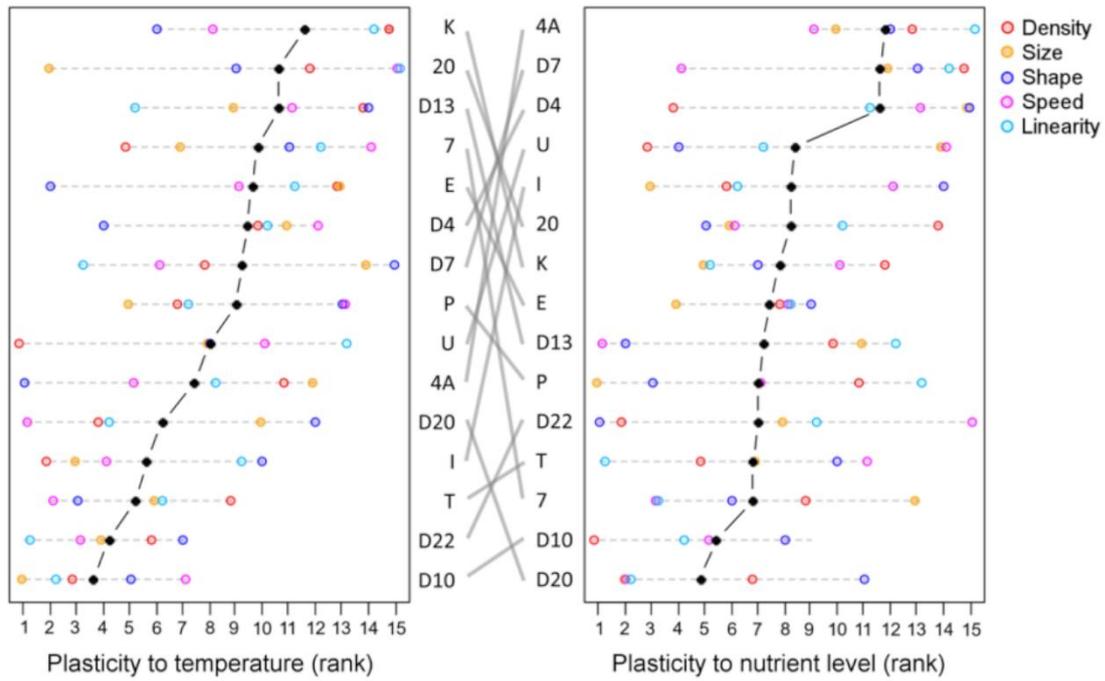
476 environmental space from the random regression mixed model.



477

478 **Figure 4:** Values of variances and co-variances of the five phenotypic traits (Den: density, Siz: size,
 479 Sha: shape, Spe: speed and Lin: linearity) for increasing values of temperatures **(A)** and for increasing
 480 values of nutrient concentration **(B)**, with the average values (solid line). **(C)** Effective number of
 481 dimensions of the G-matrices (n_D) for the 25 environmental combinations, with the effective number
 482 of dimensions for the same G-matrices with covariances forced at 0 (n_D' in parentheses) as the
 483 maximum dimensionality adjusted for heterogeneous variance in the five traits. The colours
 484 correspond to the $((n_D - 1) - (n_D' - 1)) / (n_D' - 1)$ index, expressing whether dimensionality reduction is
 485 entirely due to heterogeneous variances (index=0) or to strong covariances (index=-1).

486



487

488

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

494

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