Phenotypic integration does not constrain phenotypic plasticity: differential plasticity of traits is associated to their integration across environments

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Summary

• Understanding constraints to phenotypic plasticity is key given its role on the response of organisms to environmental change. It has been suggested that phenotypic integration, the structure of trait covariation, could limit trait plasticity. However, the relationship between plasticity and integration is far from resolved.

• Using a database of functional plasticity to drought of a Mediterranean shrub that included 20 ecophysiological traits, we assessed environmentally-induced changes in phenotypic integration and whether integration constrained the expression of plasticity, accounting for the within-environment phenotypic variation of traits. Furthermore, we provide the first test of the association between differential trait plasticity and trait integration across an optimum and a stressful environment.

• Phenotypic plasticity was positively associated with phenotypic integration in both environments, but this relationship was lost when phenotypic variation was considered. The similarity in the plastic response of two traits predicted their integration across environments, with integrated traits having more similar plasticity. Such variation in the plasticity of traits partly explained the lower phenotypic integration found in the stressful environment.

• We found no evidence that integration may constitute an internal constraint to plasticity. Rather, we present the first empirical demonstration that differences in plastic responses may involve a major reorganization of the relationships among traits, and challenge the notion that stress generally induces a tighter phenotype.

Introduction

Phenotypic plasticity, the ability of a genotype to express different phenotypes across environments, is a key mechanism to accommodate environmental heterogeneity. Due to its well-documented role on phenotypic evolution and the adaptation of organisms to rapid environmental change (Nicotra *et al.*, 2010; Matesanz & Valladares, 2014; Merilä & Hendry, 2014; Valladares *et al.*, 2014; Matesanz & Ramírez-Valiente, 2019), a strong research effort has been devoted to identifying potential constraints to the expression of plasticity and its evolution (Van Kleunen & Fischer, 2005; Valladares *et al.*, 2007; Auld *et al.*, 2010; Matesanz *et al.*, 2010; Murren *et al.*, 2015). A particularly elusive issue in this context is whether phenotypic integration – the pattern of functional, developmental and/or genetic correlation among different traits in a given organism (Pigliucci, 2003) – also constitutes an internal constraint to plasticity.

Phenotypic integration and plasticity may be related in two major ways. First, several authors have suggested that the expression of plasticity for a given trait may be limited by its overall integration, e.g. by the number of significant correlation with other traits, following the assumption that linkage with other traits limits a trait's range of variation (Schlichting, 1989a; Gianoli, 2001; Valladares *et al.*, 2007; Matesanz *et al.*, 2010). Under this hypothesis, more integrated traits would be expected to be less plastic (Fig. 1). Over a decade ago, Gianoli & Palacio-López (2009) showed, for the first time, a negative relationship between the number of significant phenotypic correlations (see Glossary in Supporting Information Table S1) of each trait and two measures of trait plasticity in response to water and light variations in two perennial plants. The authors concluded that, in certain circumstances, plasticity and integration could be alternative mechanisms to cope with stress. However, although often assumed to be a general pattern (see e.g. Zimmermann *et al.*, 2016; Vázquez *et al.*, 2017), few studies have experimentally tested the occurrence of a functional trade-off between trait plasticity and trait integration.

Second, differential plasticity (Table S1) between traits could affect the degree of across-environment integration of the
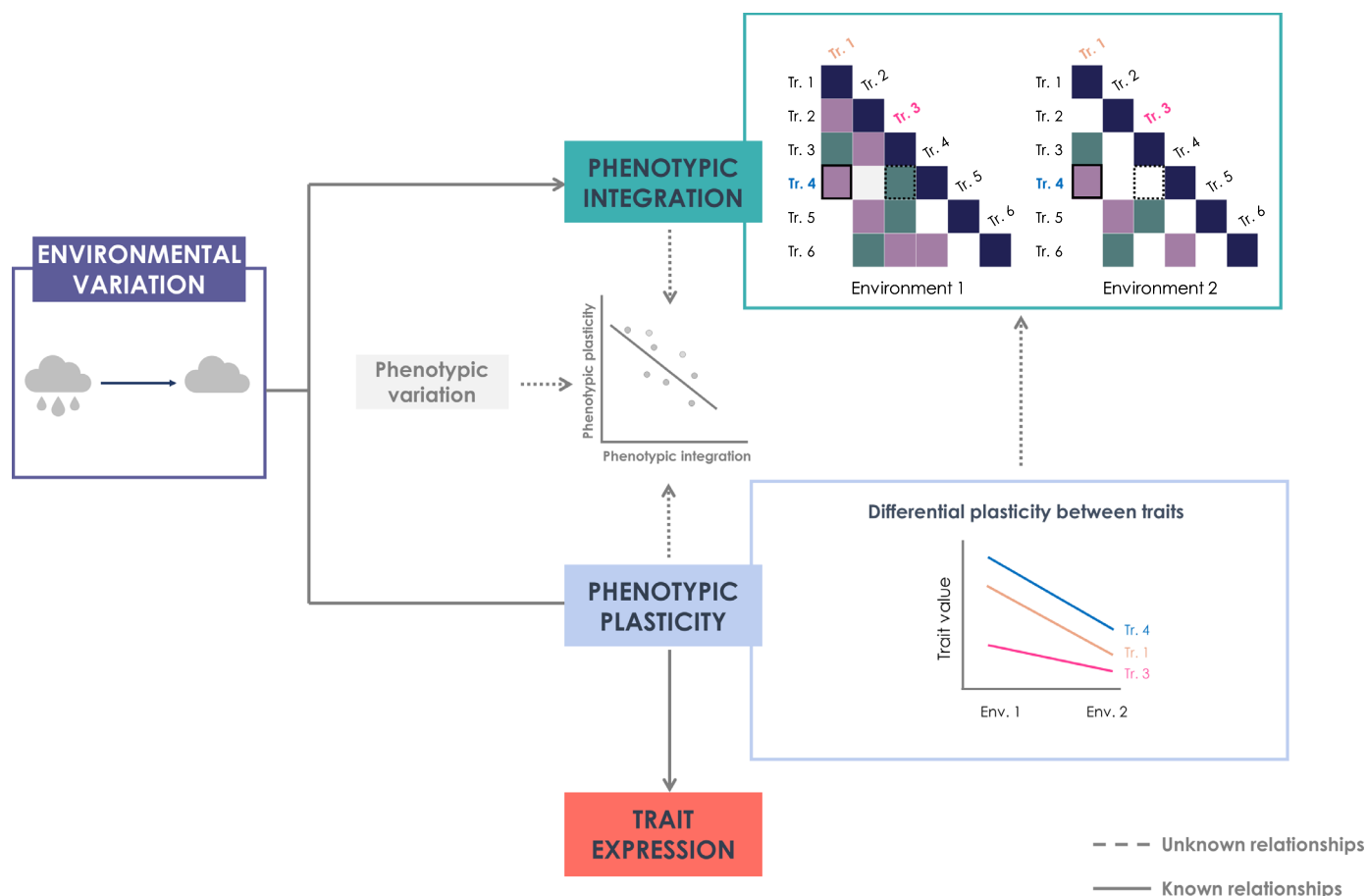
Fig. 1 The interplay between phenotypic plasticity and integration. Varying environmental conditions can influence trait expression through phenotypic plasticity. Environmental change may also alter the patterns of trait covariation, i.e. phenotypic integration, in different environments, either decreasing or increasing integration under stress (plasticity of phenotypic integration). The green box shows trait correlation matrices in two different environments, where coloured cells indicate significant correlations (of different sign) between traits. Such integration among traits, assessed here as the overall number of significant correlations of one trait to all others in a given environment, may limit trait plasticity if linkage with other traits constrains each trait's range of variation, resulting in lower plasticity of highly integrated (i.e. correlated) traits (central plot). Differences in the level of plasticity of correlated traits (blue box) may influence phenotypic integration if similar plasticity between traits contributes to maintain their integration across environments. Under this hypothesis, traits that are correlated in one environment and that express similar plasticity (Tr. 1 and Tr. 4) are more likely to also be correlated in a different environment (solid outline in correlation matrices) compared to traits that express different levels of plasticity (Tr. 3 and Tr. 4, dashed outline). This hypothesis has yet to be experimentally validated.

phenotype. In a seminal paper on the evolution of plasticity in plants, Schlichting (1986) proposed that when two traits respond differently to environmental change, i.e. they show differential plasticity, their phenotypic correlation is also likely to change across environments (Fig. 1). In this scenario, traits that are correlated in multiple environments are likely to have similar, correlated plasticity (Table S1). Conversely, the differential plasticity of traits may alter the genetic and/or environmental covariances between them, resulting in a change in their correlation (Schlichting, 1986; Parsons *et al.*, 2020). If differential plasticity of traits is indeed associated to a modification of the correlation structure in different environments, such changes on the relationship between traits could in turn alter the outcome of selection for those traits (Schlichting, 1986; Agrawal & Stinchcombe, 2009; Manenti *et al.*, 2016). Although the idea that patterns of trait correlations can be altered as a result of differential trait plasticity has been often suggested (Parsons *et al.*, 2020, and references

cited therein), experimental support to this hypothesis is to date lacking.

A better understanding of the relationship between phenotypic plasticity and integration could shed light on patterns of environmental variation of phenotypic integration, i.e. on the plasticity of phenotypic integration (Table S1). It is clearly established that the environment may not only affect the expression of individual traits, but also the magnitude and nature of trait covariation (Wood & Brodie, 2015). However, the direction, and importantly, the ecological and evolutionary implications of such plasticity of integration (Fig. 1) is not yet understood. Several authors have suggested that stress should lead to higher phenotypic integration, if a tighter and more coordinated phenotype is favoured when resources are limiting (Schlichting, 1986; Gianoli, 2004; Gianoli & Palacio-López, 2009). However, the adaptive value of increased integration in stress is yet to be determined (Pigliucci, 2003; Merilä & Björklund, 2004; but see Benavides

et al., 2021). Indeed, studies showing the adaptive value of phenotypic integration, regardless of stress level, are very scarce (Damián *et al.*, 2020). Conversely, lower integration in stressful environments could arise if an adaptive response to such stress involves differential plasticity of individual traits. However, to our knowledge, whether integration increases or decreases with stress has yet to be assessed in the light of the variation in plasticity between traits.

Here, we delved into the relationship between phenotypic plasticity and phenotypic integration in plants (Fig. 1), and its evolutionary consequences. We used a database of functional plasticity of a Mediterranean shrub that gathers data from 20 morphological, phenological, allocation and reproductive traits in individuals experimentally grown in a favourable and a stressful environment. Initially, we compared the phenotypic correlation matrices between environments to evaluate the flexibility of phenotypic integration. Then, we explored whether the expression of phenotypic plasticity of each trait is constrained by its overall level of integration with other traits. Finally, we measured the difference in plasticity for pairs of correlated traits and assessed its effect on their phenotypic integration across environments. Because the range of values that individual traits exhibit within environments (i.e. within-environment phenotypic variation, Table S1) may affect both their integration and degree of plastic response, we also considered the role of trait variation in the relationship between plasticity and integration. We asked the following questions: (i) are plasticity and integration negatively related at the trait level? (ii) is differential plasticity of correlated traits associated to their integration across environments? (iii) do environmental conditions alter patterns of variation of phenotypic integration, i.e. is there plasticity of integration, and if so, does phenotypic integration increase in a stressful environment? and (iv) does within-environment trait variation modulate the relationship between plasticity and integration? We discussed the consequences of our findings for the expression and evolution of phenotypic plasticity.

Materials and Methods

Functional plasticity database

As part of a multitaxon project examining plastic responses to abiotic stress and quantitative genetic variation in Mediterranean gypsum endemics, we investigated responses to drought in a wide selection of functional traits in populations of the perennial shrub *Lepidium subulatum* (details of the complete experiment may be found in Matesanz *et al.*, 2020). Here, we use this database to explore the relationship between phenotypic plasticity and phenotypic integration.

Lepidium subulatum L. (Brassicaceae) is a small Mediterranean shrub specialized on gypsum soils occurring in eastern Spain and North Africa, where it experiences substantial climatic variation. For this experiment, seeds from 14 to 16 maternal plants per population were collected from four populations distributed along a precipitation and temperature gradient in the centre of its distribution range (Matesanz *et al.*, 2020). The experiment was

performed in the outdoors CULTIVE facilities at Universidad Rey Juan Carlos (URJC, Madrid, Spain). Seeds from each maternal family were sown in 6 L pots filled with gypsum soil. After two years of growth, in the spring of 2017, three half-siblings per family were assigned to each of two experimental treatments of contrasting water availability ($n = 360$ plants). The experimental treatments were implemented in purpose-built rain exclusion structures that eliminated all natural precipitation. We simulated two contrasting and realistic watering treatments that reflect the soil moisture variation occurring in natural conditions: well-watered (hereafter favourable environment) and drought (hereafter stressful environment). In the favourable environment, plants were kept at field capacity ($\approx 25\%$ soil water content (SWC) for our soil). In the stressful environment, plants were kept at 50% of field capacity ($\approx 14\%$ SWC). The watering treatments were implemented by modifying the number and duration of watering events. In each of them, drip irrigation was applied on a pot-level basis by pressure-compensating emitters. The watering treatments lasted three months, and ended when plants in the favourable environment began to senesce.

Data collection

We measured 20 functional traits. Plant height was measured at the onset and end of the watering treatments in all plants ($n = 360$). From these, we calculated relative growth rate (RGR) $= (\log_e S_2 - \log_e S_1)/T_{2\text{-}1}$, where S_1 and S_2 are plant height at time 1 and time 2, respectively, and $T_{2\text{-}1}$ is the time elapsed between the two measurements. After three months since the onset of the watering treatments, midday photochemical efficiency was measured in all plants with a portable pulse-modulated fluorometer (FMS2, Hansatech, King's Lynn, UK). Measurements were taken from 13:00 to 15:00 in one leaf previously adapted to dark for 30′ with leaf clips during three consecutive sunny days. Minimal fluorescence (F_o) and maximal fluorescence (F_m) were used to calculate photochemical efficiency as $F_v/F_m = (F_m - F_o)/F_m$, where F_v is the difference between F_m and F_o.

After three months in treatment, eight nonsenescent leaves per plant were randomly selected and scanned. Then, leaf length and leaf area were calculated in Adobe PHOTOSHOP (Adobe Systems Inc., San Jose, CA, USA). Leaves were oven-dried for 48 h at 60°C and weighed. Specific leaf area (SLA) was estimated as the ratio of the one-side area of a fresh leaf divided by its oven-dry mass. Aboveground tissues of each plant were harvested and oven-dried, separated and weighed (leaf and stem biomass). Leaves were separated from the stems, oven-dried and weighed in a Kern ABJ 120-4M analytical balance (Kern & Sohn GmbH, Albstadt, Germany). Aboveground biomass was calculated as the sum of leaf and stem biomass. Total plant estimated leaf area (TELA) was calculated as SLA × leaf biomass, and leaf : stem ratio was calculated as leaf biomass divided by stem biomass. Finally, for one randomly-selected plant per family and treatment ($n = 120$), roots were thoroughly washed, oven-dried and weighed. For this subset of plants, we calculated root : leaf ratio as root biomass/leaf biomass, and total biomass as the sum of leaf, stem and root biomass.

Reproductive phenology was monitored in all plants with 2–3 censuses per week throughout the experiment. We considered three different phenological events: onset of flower bud formation (flower buds visible to the naked eye), onset of flowering (appearance of open flowers), and onset of fruiting (green fruits visible to the naked eye). Before harvesting, the percentage of senescent leaves was visually estimated simultaneously by two observers in all plants. At the end of the experiment, we counted the number of inflorescences of all plants that had reproduced during the experiment ($n = 287$ reproductive plants). We randomly selected two inflorescences per plant and measured its size and the number of flowers. We then collected and weighed all reproductive biomass of each plant, and thoroughly cleaned the inflorescences to separate mature seeds. For these plants ($n = 155$), we individually weighed 3–5 seeds per plant in a microbalance.

Metrics of within-environment phenotypic variation, phenotypic integration and phenotypic plasticity

We first computed the matrices of pairwise trait correlations in each environment ($C_{ij,f}$ and $C_{ij,s}$ in the favourable and stressful environments, respectively). Pairwise Pearson correlation coefficients were computed using function *cor* (package CORRPLOT; Wei & Simko, 2017). Trait values were scaled and transformed (squared root and log) as needed to improve normality. To evaluate the significance of the correlations, we implemented permutation tests using function *perm.cor.test* (package JMUOUTLIER; Garren, 2019). Trait values were shuffled 20 000 times, and pairwise correlations were calculated from each randomization. This generated a permutation distribution of correlation coefficients from which *P*-values were calculated. Correlation matrices were calculated: (1) on a plant-level basis (phenotypic correlations) and (2) using family means. Both metrics resulted in very similar matrices, and indeed, previous studies showed that phenotypic correlations are reasonable estimates of their genetic counterparts (Cheverud, 1988; Waitt & Levin, 1998). Therefore, we used phenotypic correlations that allowed the inclusion of all measured traits. A second set of phenotypic correlation matrices was computed where significance values were corrected for multiple comparisons using false discovery rate (Benjamini & Hochberg, 1995).

The overall phenotypic integration for each trait (PI_i) was computed as the number of significant correlations with all other traits (see e.g. Pigliucci & Marlow, 2001; Murren *et al.*, 2002; Gianoli, 2004; Gianoli & Palacio-López, 2009 for other studies using this metric of phenotypic integration). We computed PI_i both for the favourable ($PI_{i,f}$) as well as for the stressful environments ($PI_{i,s}$). It has been proposed that the statistical significance (*P*-values) of a correlation may be biased by sample size, and significant correlations can be found if the amount of data is sufficiently large, even if the association between two traits is low (García-Verdugo *et al.*, 2009, and references cited therein). To avoid this bias, we also computed $PI_{i,f}$ and $PI_{i,s}$ as the average Pearson correlation coefficients of one trait to all others, which avoids sample size bias, and as the geometric mean of the squared coefficients of correlation, as suggested by Pigliucci *et al.* (1991).

Importantly, all metrics of phenotypic integation in each environment were highly correlated ($P < 0.001$), indicating that our chosen measure of trait-level integration is unbiased. Phenotypic plasticity (PP_i) was estimated as the percentage of change in trait expression in the stressful environment compared to the favourable environment. For this, we calculated the phenotypic plasticity index (Valladares *et al.*, 2006) as $100 \times$ (absolute mean differences between environments/mean in favourable environment), which is appropriate when the goal is to compare plasticity among traits (Valladares *et al.*, 2006). Note that the calculations of the index of plasticity using trait means in the stressful environment as the denominator did not alter the results (data not shown). Finally, within-environment phenotypic variation for each trait and environment ($PV_{i,f}$ and $PV_{i,s}$) was calculated as the coefficient of variation of each trait, as $100 \times$ (standard deviation/trait mean).

Relationship between phenotypic plasticity and phenotypic integration

We explored the relationship between phenotypic plasticity and phenotypic integration in the two main ways depicted in the Introduction. Initially, we evaluated whether the plastic response of each trait (PP_i) was related to its overall phenotypic integration (PI_i). For this, we used standardized major axis regression using function *sma* from package SMATR (Warton *et al.*, 2006; Warton *et al.*, 2012). This tool finds the line of best fit between two variables, i.e. the purpose is to characterize the relationship between two variables rather than predicting one from the other. The first study assessing this relationship (Gianoli & Palacio-López, 2009) used the phenotypic integration data from the stressful environment, arguing that it is in such environments where the target functional phenotype, the phenotypic expression that allows to maintain function in that environment, is attained. To avoid this arbitrariness, we fitted a standardized major axis separately for each environment, using the plasticity indices calculated for each trait (PP_i) and the phenotypic integration values computed for each environment ($PI_{i,f}$ and $PI_{i,s}$). Then, to check whether the relationship between plasticity and integration differed between environments, we tested for common slopes and elevations of the environment-specific fitted lines (Warton *et al.*, 2012). We computed 95% BCa (bias-corrected and accelerated) bootstrap intervals (Davison & Hinkley, 1997) based on 2000 replications, using functions *boot* and *boot.ci* from package BOOT (Canty & Ripley, 2021).

Furthermore, we assessed whether the relationship between phenotypic plasticity (PP_i) and integration (PI_i) was indirectly mediated by the within-environment phenotypic variation (PV_i) of traits. Specifically, we used commonality analysis to calculate the unique and shared effect of phenotypic integration and phenotypic variation on plasticity indices and their significance. This analysis decomposes the variance of R^2 into unique and common (shared) effects of predictors, improving the partition of the variance of the study variable when predictors are correlated (Ray-Mukherjee *et al.*, 2014). We performed a commonality analysis in each environment, using function *regr* from package YHAT

(Nimon *et al.*, 2013). PP_i and PV_i values were log-transformed before analyses.

Next, we tested whether differences (or similarities) in plasticity between two traits are related to their integration, i.e. correlation, across environments, with the hypothesis that correlated traits in the two environments also show correlated plasticity. To do so, we initially computed the matrix of differences in phenotypic plasticity among traits (ΔPP_{ij}, plasticity difference matrix). This difference was estimated by the fitted coefficient of the trait-by-environment interaction (Trait × Environment) in a linear model fitted with function *lm* testing the effect of the environment on trait values. A significant Trait × Environment interaction indicates that two given traits show differential plasticity, with nonparallel norms of reaction (Fig. 1). The larger the estimate, the larger the difference in plasticity among the two traits. Because we were interested in the absolute value of the differences in plasticity rather than the direction of the plastic response (i.e. whether both traits show high or low plasticity rather than whether they increase or decrease their values), trait values were transformed by changing their sign as needed to maintain the same direction of the response. We used the estimate of the coefficient for the interaction term as a measure of the differences in plasticity, since it provides a continuous measure of the strength of the interaction, i.e. a quantitative measure of plasticity differences between any two traits. We repeated our analyses with matrices built using different metrics of the size effect of the interaction, namely the *P*-value of the interaction term, the *F* statistic and the sum of squares, obtaining very similar results. Traits were scaled and transformed as needed to meet normality assumptions.

Then, we calculated the change in phenotypic integration for each pair of traits by comparing their correlation in the two environments. Specifically, we computed the matrix of similarities of pairwise trait integration between environments (ΔC_{ij}, integration similarity matrix) by comparing the matrices of trait correlations in the favourable and stressful environments ($C_{ij,f}$, $C_{ij,s}$). Then, the cells of ΔC_{ij} were set to 1 if the traits i and j were significantly correlated in both environments; to 0 if the traits were significantly correlated only in one environment and to −1 if the traits were not correlated in either environment.

Finally, the plasticity difference matrix (ΔPP_{ij}) and the integration similarity matrix (ΔC_{ij}) were transformed into vectors, and a generalized linear model was fitted (function *glm*, family = binomial) to test the association between differences in trait plasticity on the maintenance of trait correlation. Because we were only interested in correlated traits, we did not consider trait pairs that were not correlated in either environment (cells coded as −1 in ΔC_{ij}). A significant and negative effect of the differences in plasticity would indicate that the more different the plasticity of two traits, the more likely it is that they will change their correlation across environments. Again, we computed 95% BCa bootstrap intervals based on 2000 replications.

Plasticity of phenotypic integration

To assess the effect of the environment on phenotypic integration, i.e. the plasticity of phenotypic integration, we compared the correlation matrices of each environment ($C_{ij,f}$ and $C_{ij,s}$) using four different approaches. Initially, we calculated the edge density in each environment, assessed as the ratio between the number of significant correlations (edges) and all possible pairwise trait combinations. Next, we tested differences between the matrices using function *cortest.normal* (package PSYCH; Revelle & Revelle, 2015), which computes a chi-square statistic based on the difference between matrices under the hypothesis that they are equal. Because matrices may not be equal but still have similarities (Roff *et al.*, 2012), we then used common principal components (CPCs) based on Flury's hierarchy of hypotheses testing (Phillips & Arnold, 1999), which allows a much more complex comparison of the correlation structure in different environments (Pigliucci, 2003; Roff *et al.*, 2012). Specifically, this method can test the following hypotheses of decreasing matrix relatedness: (1) matrices are identical, sharing both principal components (eigenvectors) and eigenvalues, (2) matrices are proportional, when the matrices share their components but the eigenvalues differ by a constant, (3) matrices share all eigenvectors but not eigenvalues (CPCs), (4) matrices share some eigenvectors (partial CPC) and (5) matrices are unrelated, sharing no principal component in common. We used both the jump-up and the model-building approaches to matrix comparisons (Phillips & Arnold, 1999). In the jump-up approach, different levels of the Flury's hierarchy are compared to 'unrelated' structure. In the model building approach, the Akaike information criterion (AIC) is used to determine which level of shared structure in the hierarchy best fits the data. CPC analyses were performed using package CPC (Pepler, 2019). Finally, the magnitude and statistical significance of phenotypic integration in each environment was further assessed using the phenotypic integration index INT (Wagner, 1984; Cheverud *et al.*, 1989) which calculates the variance of the eigenvalues (λ_i, INT = Var [λ_i], $i = 1, 20$) of the matrices of pairwise trait correlations in each environment (INT_f and INT_s). High variance among eigenvalues indicates high phenotypic integration (Damián *et al.*, 2020). Confidence intervals of INT_f and INT_s at 95% and 99% were obtained by bootstrapping (20 000 replications) the matrices of trait correlations and calculating the variance of the eigenvalues for each bootstrap (see Herrera *et al.*, 2002). We considered nonoverlapping confidence intervals between environments as evidence of significant plasticity of phenotypic integration.

Results

Relationship between phenotypic plasticity and phenotypic integration

We found a positive relationship between phenotypic plasticity and phenotypic integration in both environments (Fig. 2). The traits that showed higher plasticity, assessed by the phenotypic plasticity index, had more significant phenotypic correlations with other traits in both environments. Although this relationship was stronger in the favourable environment ($R^2 = 0.35$, $P = 0.009$) than in the stressful environment ($R^2 = 0.212$, $P = 0.041$), neither the slope of both major axes (Likelihood ratio

statistic: 0.0822, df = 1, $P < 0.774$) nor their elevation (Wald statistic: 1.717, df = 1, $P < 0.190$) were significantly different between environments. This relationship was still significant in the favourable environment when integration data were corrected for multiple comparisons (Table S2).

Variance partition analysis (commonality analysis) showed that phenotypic integration and within-environment phenotypic variation jointly explained more than 60% of the variance in phenotypic plasticity in the favourable environment (Fig. 3). However, the unique, i.e. not shared, variance in plasticity explained by phenotypic integration was only 4.8%, while the unique contribution of phenotypic variation was much higher (32.1%). Accordingly, the model including both the effect of phenotypic variation and integration on plasticity showed a significant effect of phenotypic variation ($F = 15.77$, $P = 0.001$; Supporting Information Fig. S1) on plasticity but not of integration ($F = 3.21$, $P = 0.091$). A similar pattern was found in the stressful environment (Fig. 3). The fraction of variance in plasticity uniquely explained by phenotypic integration was low (4%) and not significant ($F = 2.22$, $P = 0.15$) compared to the unique higher effect of phenotypic variation on plasticity (23.2% explained variance, $F = 7.98$, $P = 0.011$; Fig. S1). Phenotypic integration was not significantly related to phenotypic variation in the stressful environment ($P = 0.146$), and only marginally so in the favourable environment ($P = 0.050$).

The model testing the association between the differences in trait plasticity on the maintenance of trait integration across environments for all pairs of traits showed a significant and negative relationship between them ($\chi^2 = 6.7176$, $P = 0.009$; Fig. 4). Traits with differential plasticity in response to the stressful environment (i.e. nonparallel norms of reaction, significant Trait × Environment interaction) were more likely to change their integration (whether they were correlated or not) between environments compared to traits with similar plasticity, and, conversely, traits that were correlated in both environments tended to express similar, correlated plasticity. This relationship was also significant when other metrics (P-value of the interaction term, the F statistic and the sum of squares) of the differences in plasticity were used (Table S2).
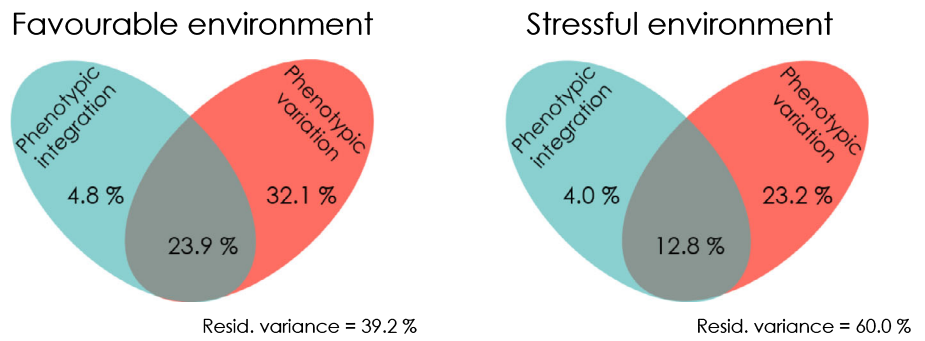
Plasticity of phenotypic integration

The environment significantly affected the structure of the matrices of trait correlations. Initially, the test of equality of the correlation matrices showed that they were significantly different ($\chi^2 = 1567.39$, df = 380, $P < 0.0001$). Flury's CPC analysis provided further evidence for differences in the correlation matrices between environments. This analysis showed that the matrices were different at several levels of structure (equality, proportionality and all principal components shared), according to the
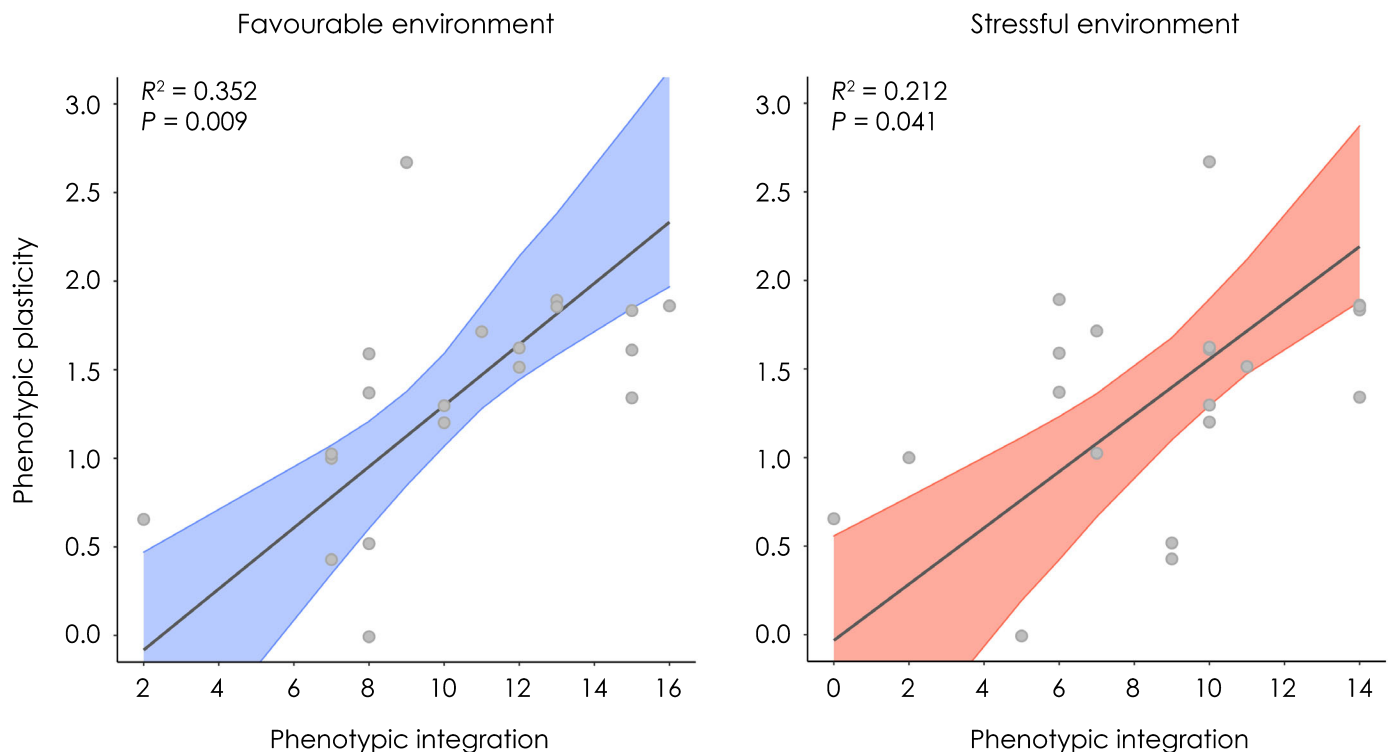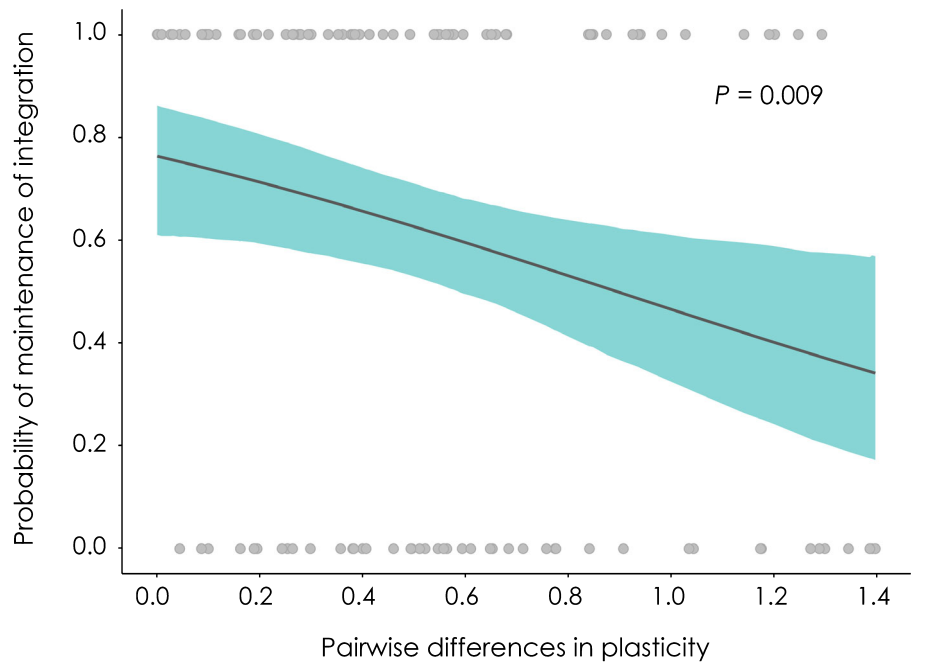

Fig. 2 Relationship between phenotypic plasticity of a given trait and number of significant correlations with other traits, phenotypic integration, assessed by major axis regression. For each trait, phenotypic plasticity was calculated as the percentage of change in mean trait expression in plants growing under two treatments of water availability (well-watered, favourable environment, and drought, stressful environment). Values of log (% change) are shown. Each point in the regression analysis corresponds to a single trait ($n = 20$). The coefficient of determination of the regression (R^2) and P-value are shown. The shade in each panel indicates 95% BCa (bias-corrected and accelerated) bootstrap intervals based on 2000 replications.

Variation in plasticity

Fig. 3 Venn diagrams showing results of the commonality analyses to partition the variance of phenotypic plasticity. Ovals show the unique contribution and the intersection the shared contribution of phenotypic integration (number of trait correlations) and phenotypic variation (coefficient of variation) on the variance of phenotypic plasticity in both a favourable environment and a stressful environment.

Fig. 4 Model testing the relationship between the differences in phenotypic plasticity and the change in phenotypic integration. Change in phenotypic integration was coded as 1 (no change in integration) and 0 (traits correlated in one environment but not in the other). Differences in plasticity between traits were estimated as the fitted coefficient for the Trait × Environment interaction in a linear model relating trait values to environmental conditions for each pair of traits. The shade indicates 95% BCa (bias-corrected and accelerated) bootstrap intervals based on 2000 replications. See text for details.


jump-up approach, and that the most likely model was that they were unrelated, according to lowest AIC in the model-building approach (Table S3).

These results reflected both differences in the total number of correlations in each environment and the identity of correlated traits. Edge density, the proportion of significant correlations, was higher in the favourable environment compared to the stressful environment. Similarly, the magnitude of phenotypic integration estimated by INT was significantly higher in the favourable environment compared to the stressful environment, as shown by nonoverlapping confidence intervals (CIs) (INT_f = 2.221, 95% CI 1.948–2.526, 99% CI 1.871–2.645; INT_s = 1.478, 95% CI 1.302–1.680, 99% CI 1.258–1.748). Of the 190 pairwise correlations (20 × 20 traits), 103 (54.2%) were significant in the favourable environment ($P < 0.05$), and 87 (45.7%) in the stressful treatment (Fig. 5). Of these correlations, 31 were unique (i.e. only present) in the favourable environment and 15 in the stressful environment. Similar proportions of positive and negative trait correlations were found in both environments (62.2–67.8%

positive correlations vs 37.8–32.2% negative correlations, respectively). Correction for multiple comparisons using false discovery rate had a low impact on the correlation matrices, affecting 7.8% and 10.3% of the total correlations in the favourable and stressful environment, respectively (Fig. S2).

Discussion

Phenotypic plasticity was positively associated with phenotypic integration in both environments. Traits that were more integrated, i.e. linked to more traits, expressed higher plasticity in response to the stressful environment (Fig. 2). However, the relationship between phenotypic integration and plasticity was no longer significant when accounting for the within-environment phenotypic variation of each trait (Fig. 3). The latter did not indicate, however, that plasticity and integration were completely independent mechanisms. We empirically show for the first time that the similarity in the plastic response of two traits is associated to their integration across environments, with traits with more
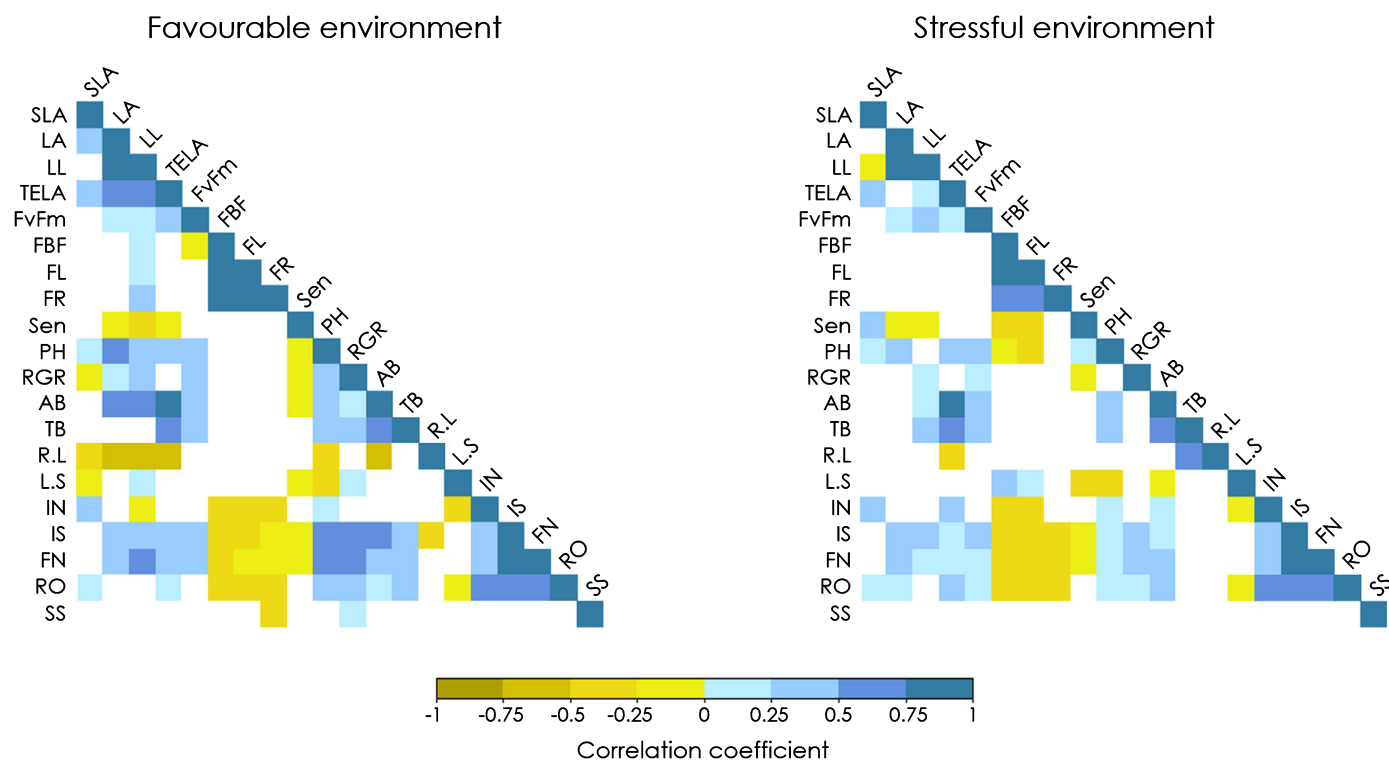
Fig. 5 Correlation matrix showing Pearson's pairwise correlation coefficients for traits measured in a favourable environment (left) and a stressful environment (right). Only significant (*P* < 0.05) phenotypic correlations are coloured. Significance of the correlation coefficients was based on 20 000 permutations. Traits were transformed to approximate normal distributions. SLA, specific leaf area; LA, leaf area; LL, leaf length; TELA, plant-level leaf area; FvFm, photochemical efficiency; FBF, onset of flower bud formation; FL, onset of flowering; FR, onset of fruiting; Sen, percentage of senescent leaves; PH, plant height; RGR, relative growth rate; AB, aboveground biomass; TB, total biomass; R.L, root : leaf ratio; L.S, leaf : stem ratio; IN, inflorescence number; IS, inflorescence size; FN, flower number; RO, reproductive output; SS, seed mass.

similar plasticity having a higher probability of being correlated in both environments (Fig. 4). Altogether, our results indicate that plasticity is not constrained by integration, but rather, differential plasticity between traits may result in lower integration in response to stress. Furthermore, this study provides novel evidence on the relationship between the phenotypic variation of traits within environments and their degree of response to stress, which, surprisingly, was a much better predictor of plasticity than integration.

The complex relationship between phenotypic plasticity and integration

A few authors have suggested that different environmental sensitivity, i.e. plasticity, of traits may lead to changes in their integration across environments (Parsons *et al.*, 2020, and references cited therein). In particular, more than three decades ago, Schlichting (1986, 1989a) proposed that when two traits respond differently to environmental change, one being more plastic than the other, the phenotypic correlation between them in different environments is likely to change. However, experimental evidence on the consequences of differential plasticity on patterns of phenotypic integration between environments was to date lacking. Here, we demonstrated that when two traits were correlated in one environment and they showed similar plasticity in response to stress, i.e. they showed correlated plasticity, they were

more likely to also be correlated in the second environment. Conversely, significant differences between traits in their degree of response to environmental variation (evidenced by a trait-by-environment interaction) were associated to a change of integration between environments, often involving a loss of correlation between traits in the stressful environment. For instance, in the favourable environment, individual leaf area showed a positive association with relative growth rate (see also Padilla *et al.*, 2009), indicating that plants with larger leaves also grew faster. However, in response to water stress, plants reduced the area of individual leaves to a larger extent (i.e. greater leaf area plasticity) than the observed reduction in growth. Such difference in the plasticity of the two traits was coupled to a lack of significant correlation between them in stressful conditions. Importantly, the link between differences in plasticity and changes in integration was observed not only for morphological and growth traits but also for pairs of all functional categories (Fig. S3). To our knowledge, this is the first study showing a significant relationship between the differential plasticity of traits and the integration of the phenotype across environments (Fig. 1).

Our findings have significant evolutionary implications. Previous studies reported that integrated traits often show correlated, i.e. integrated, plasticity (Schlichting, 1986; Schlichting & Pigliucci, 1998; Plaistow & Collin, 2014; Ellers & Liefting, 2015; Parsons *et al.*, 2020, and references cited therein). It has been argued that such plasticity integration (Schlichting & Pigliucci,

1998; Pigliucci, 2003) could constrain the expression of plasticity and its evolution (Schlichting, 1989a; Montague *et al.*, 2013) if the same correlated response is consistently expressed. However, we found that correlated traits can also show differential plasticity, often resulting in a loss of correlation in the stressful environment. Such a change in the phenotypic correlation between two traits indicates a modification of the genetic and/or environmental covariances between them (Schlichting, 1986; Sgrò & Hoffmann, 2004). We thus propose that differences in plasticity between two traits may alter trait covariances, allowing flexibility in the phenotypic correlation structure across environments. Importantly, the maintenance or loss of correlations between traits may affect the outcome of selection for those traits in different environments (Schlichting, 1986, 1989a; Agrawal & Stinchcombe, 2009; Plaistow & Collin, 2014; Manenti *et al.*, 2016). Therefore, if such changes are indeed mediated by plasticity, our results would indicate the potential for plasticity to impact selection on correlated traits.

We are far from understanding the precise mechanism underlying the association observed between differences in the plasticity of traits and changes in their phenotypic correlation. A potential explanation could be that differential plasticity between traits is coupled to among-individual differences in plasticity. For instance, two traits could differ in their plasticity without affecting integration, if all individuals respond plastically in a similar way. However, if individuals do vary in their plasticity, and the degree of plasticity that each individual expresses also differs across traits, the covariation between those traits may be altered, resulting in a change in their correlation (see also Killen *et al.*, 2013; Peiman & Robinson, 2017). Further experiments with genotypes or families differing in the expression of plasticity across traits in response to diverse environmental conditions will undoubtedly shed light on this unresolved question.

Together with the association between the maintenance of integration and differential trait plasticity, we also explored whether the overall integration of a trait (its correlations to all other traits) was related to its ability to express a plastic response. Gianoli & Palacio-López (2009) were first to report a negative relationship between the number of significant phenotypic correlations of a trait and its plasticity. Conversely, we observed a positive association between plasticity and integration, with more integrated traits, both in the stressful and favourable environment, also being more plastic. However, the unique contribution of phenotypic integration to explain variation in plasticity among traits was not significant when we accounted for the phenotypic variation of traits within environments, indicating that plasticity and integration were, in fact, unrelated at the trait level. These contrasting results indicate that plasticity is not generally constrained by integration. Indeed, the very few studies that have tested the relationship between overall trait integration and plasticity have shown remarkably varying results, with very weak support to the existence of a functional trade-off between them. For instance, a recent study found a positive relationship between plasticity and integration in three tree species occurring in Restinga-like and seasonal semideciduous tropical forests (Pireda *et al.*, 2019). Similarly, Zimmermann *et al.* (2016) found that more correlated traits showed more plasticity in response to light availability in seedlings of an invasive tree, although this positive association was only found when integration was assessed in stressful shade conditions. Conversely, a study on thermal physiology of intertidal crabs (Osores *et al.*, 2018) showed an inverse relationship between plasticity and integration, while Godoy *et al.* (2012) found that the sign of the relationship changed depending on whether morphological or physiological plasticity was considered. Notably, none of these studies considered the potential effect of the within-environment phenotypic variation of traits on the relationship between plasticity and integration, which in our case mediated the positive association observed, and they used contrasting metrics of both integration and plasticity. This indicates that the role of phenotypic integration as an internal constraint to plasticity cannot be assumed as a general pattern, as it is often invoked in the literature (see e.g. Zimmermann *et al.*, 2016; Vázquez *et al.*, 2017). Clearly, more experimental data using standardized approaches is needed to assess both the prevalence and sign of this relationship, and the environmental conditions that may drive its occurrence (Gianoli & Palacio-López, 2009).

The plasticity of phenotypic integration

Although the mechanisms are not clearly understood, experimental evidence has repeatedly shown, for both animal and plant taxa, that environmental conditions affect not only the expression of individual traits but also the covariance between them (Schlichting, 1989b; Pigliucci *et al.*, 1995; van Tienderen & van Hinsberg, 1996; Pigliucci, 2003; Sgrò & Hoffmann, 2004; Handelsman *et al.*, 2014; Plaistow & Collin, 2014; Manenti *et al.*, 2016; Cousins & Murren, 2017; Peiman & Robinson, 2017; Jonas & Navarro, 2019; Carvalho *et al.*, 2020). Our hierarchical matrix comparison showed that, when compared to the favourable environment, elements of the correlation matrix in the stressful environment were not equal, not proportional, and did not share any CPCs. The lower integration observed in stress – as evidenced by the lower edge density and lower phenotypic integration index INT – reflected both a reduction of the overall number of trait correlations and, to a lower extent, changes in the nature of trait covariation.

The differential plasticity of traits can partly explain the lower overall phenotypic integration found in the stressful environment. Specifically, two out of three cases where we found a change of integration between environments involved loss of correlation in the stressful environment, and of them, more than 80% of pairs had differential plasticity between traits (Fig. S3). This challenges the idea that phenotypic integration should increase in stress. Indeed, although this has been often suggested in the literature (Schlichting, 1986; Gianoli & Palacio-López, 2009), experimental tests have failed to offer a clear pattern, with a few studies confirming higher integration in stressful conditions (Schlichting, 1989b; Gianoli, 2004; Seguí *et al.*, 2018; Benavides *et al.*, 2021), while others showing no change of integration between environments (Pigliucci & Kolodynska, 2002a,b, 2006; Mallitt *et al.*, 2010) or even reporting lower integration in

stressful conditions, as also found here (Boucher *et al.*, 2013; García-Cervigón *et al.*, 2021; see also Cousins & Murren, 2017). We propose that, in some instances, the functional response to stress may involve differential plastic responses of multiple traits, which may in turn result in lower integration in a stressful environment. In the light of our results, we encourage researchers to expand the focus to account for the differences in plasticity between traits and how they affect trait integration, and most importantly, to assess the adaptive value of a tighter phenotype in stress (Schlichting, 1989; Gianoli & Palacio-López, 2009; Damián *et al.*, 2020), which may prove useful to further our understanding on the plasticity of phenotypic integration.

The positive association between plasticity and phenotypic variation within environments

An unexpected result of our study was the strong and positive relationship between the within-environment phenotypic variation of individual traits and their plasticity in response to the stressful environment (Fig. S1). Both phenotypic variation and plasticity are well-known to vary among traits. For a specific group of organisms reared in a set of controlled experimental environments, a common outcome is to find that some traits are more plastic than others and that some traits are more variable than others in those environments. However, the association between both aspects of trait variation – within and between environments – has rarely been established. Phenotypic variation within environments may arise from genetic differences among individuals, microenvironmental effects and developmental instability, i.e. variation among replicates of a genotype in the phenotype produced in a specific environment, also termed developmental 'noise' (Møller, 1997). In a recent study using recombinant inbred lines (RILs) of *Arabidopsis thaliana*, Tonsor *et al.* (2013) found a significant link between trait plasticity and both the coefficients of genetic and environmental variation of those traits within environments, and a genetic correlation between developmental instability and RIL plasticity at the trait level (see also van Kleunen *et al.*, 2000; Valladares *et al.*, 2002; Ørsted *et al.*, 2018). In our study, we cannot separate which component of within-environment variation is most correlated with trait plasticity. However, as we observed this positive pattern in both the favourable and stressful environments, our results suggest the intriguing possibility that the plastic response of specific traits could be predicted from its range of variation expressed in a single environment.

Conclusions

Our study provides new insight on the multifaceted relationship between the flexibility – plasticity – and the coherence – integration – of the phenotype. Overall, we found no evidence that integration may constitute an internal constraint to plasticity. Rather, by assessing how differences in plasticity between traits affect their integration across environments, we present the first empirical demonstration that the differential expression of

plasticity may involve a major reorganization of the relationships among traits (Schlichting, 1986, 1989a). Differences in the degree of response among traits were coupled to a significant loss of integration in the stressful environment, challenging the notion that stress generally induces a tighter phenotype. Future studies should focus on identifying the mechanisms governing the link between plasticity and integration, and most importantly, on how such interplay may affect the adaptive value of these key aspects of organisms' function.

Author contributions

SM and AE conceived the idea. MB-S, MR-M and SM collected the data. MC, RB, MB-S, MR-M and SM analysed the data. SM wrote the manuscript with input from all other authors.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request at http://repositories.biodiversos.org/Matesanz_S/.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Relationship between within-environment phenotypic variation and trait plasticity.

Fig. S2 False discovery rate-corrected Pearson pairwise correlations assessed in favourable and stressful environments.

Fig. S3 Matrix of changes in phenotypic integration.

Table S1 Glossary of the terms used throughout the manuscript.

Table S2 Results of the standardized major axis regressions using false discovery rate-corrected values.

Table S3 Flury common principal component (CPC) tests for correlation matrix comparisons.

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