

Phenotypic integration in three *Lamium* species' response to light and density

Nataša Barišić Klisarić*, Danijela Miljković, Stevan Avramov, Uroš Živković and Aleksej Tarasjev

Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia

*Corresponding author: natasa@ibiss.bg.ac.rs

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Abstract: Phenotypic correlations were calculated and the levels of phenotypic integration of three congeneric species, annual *Lamium purpureum* and perennial *Lamium album* and *Lamium maculatum*, were estimated. Calculations were performed separately for different stages of development during transplantation under different density and light treatments. *L. purpureum* exhibited greater integration in comparison to the other two species before exposure to environmental treatments, as well as greater variability in phenotypic correlations in response to light and density. The perennial species possessed similar correlation patterns in comparison to annual *L. purpureum*, becoming more similar to *L. purpureum* at later stages of development. The effects of density and light intensity were dependent on the specific combination of factors, as well as on the species under study. Density effects in *L. purpureum* were also habitat- and population-specific. A greater difference in response to density treatments between habitats than between populations was detected. Overall, phenotypes become more integrated during ontogeny, while dissimilarities in phenotypic correlations among species and between populations were more related to ecological than to phylogenetic differences.

Keywords: *Lamium album*; *Lamium maculatum*; *Lamium purpureum*; phenotypic correlations; static integration

INTRODUCTION

Correlations among traits are a consequence of the coordination of multiple pathways underlying those traits at different levels [1]. Understanding morphological correlation/covariation has been a major challenge in ecology and evolution, and a comparative approach can help in this pursuit [2]. Correlation patterns of the multivariate phenotype can be observed ontogenetically or at genotype, population, species or higher classification levels and in response to different environmental treatments [3-7]. Trait correlations examined at levels above the species are so-called "evolutionary correlations" because they reflect macroecological and macroevolutionary processes [2]. Trait correlations below species levels are referred to as "static correlations" and can be a consequence of genetic, developmental, functional, physiological or ecological causes [2,8,9].

Phenotypic integration is defined by the magnitudes and patterns of morphological trait correlation/covariation [10,11]. It can be seen both as an adap-

tation to a set of environmental conditions, and as a constraint to evolution [10,12]. Integration can be flexible and modified by natural selection, but interconnection among traits can also restrain evolutionary changes [10,13]. Constraints can even play a positive role by directing existing variations towards the position where selective changes are likely to arise, or by creating "byproducts" of evolution that could be a starting point for some new adaptations [14,15].

It has been proposed that integration should be higher under environmental stress [16]. Plants are exposed to stressful environments such as changes in light, temperature, moisture, salinity and the presence of heavy metals that surpass daily and seasonal cycles [17,18]. Variations in light intensity and spectral quality are of essential importance for plants [19]. Neighbor density variation often leads to a particular set of plastic changes known as "shade avoidance syndrome", which includes stem elongation, reduction of branching and acceleration of flowering [20,21]. A reduction in the red to the far-red ratio of light (R:FR) induces

these responses as a cue for neighbor proximity [22]. Lowering light intensity, however, can lessen the rate of plant development [23].

In two previous experiments, we addressed the plasticity of traits important for light acquisition in the genus *Lamium* due to alterations in photosynthetically active radiation (PAR) perceived by the plant [24,25]. The potential for evolutionary change depends not only on single traits but also on the nature of their relationships. Therefore, in the present study, we have expanded our research to investigating the strength and pattern of trait correlations. First, we examined trait correlations in three *Lamium* species: *Lamium purpureum* (L.), *Lamium album* (L.), and *Lamium maculatum* (L.), during transplantation and growth under exposure to two light treatments (low and high) and three planting densities (1, 3 and 5 plants per pot). Additionally, we studied the correlations of traits in *L. purpureum* plants that originated from two populations (Vršac and Avala) and two habitats within populations (shade and open), under two density treatments (low and high).

Since the causes of trait correlations can change depending on the level of organization, each level can provide an additional contribution by addressing the following ecological and evolutionary issues: (i) how do the levels and patterns of phenotypic integration differ among three congeneric species during transplantation and under applied light and density treatments; (ii) how do the level and pattern of phenotypic integration differ between two populations and habitats of *L. purpureum* during transplantation and under different density treatments; (iii) whether trait correlations are shaped more by the environment or by evolutionary history.

MATERIALS AND METHODS

Species descriptions and measured traits

Three members of the mint family (Lamiaceae), *Lamium purpureum* (red dead nettle), *Lamium album* (white dead nettle) and *Lamium maculatum* (spotted dead nettle) were used. *L. purpureum* is an annual or biannual species and inhabits open habitats and forest edges [26]. *L. album* and *L. maculatum* are perennials and occupy habitats with more shade. *L. album*

prefers the most eutrophic habitats, followed by *L. purpureum*, while *L. maculatum* prefers the least eutrophic and most shaded habitats [26].

Seeds of all three species were collected from plants grown in the “Jevremovac” Botanical Garden, University of Belgrade, Serbia. Nine replicates of each species/density combination were placed in one of two light treatments and one of two blocks. Due to the smaller number of seedlings available, the second block had only 5 replicates of *L. maculatum* per density/light treatment combination. In a separate experiment, 21 replicates of each population/habitat combination of *L. purpureum* were exposed to one of two density treatments and one of four blocks. Detailed descriptions of both experimental designs are presented in previous research papers [24,25].

The first measurement (during transplantation from growth containers to pots) was performed on juvenile plants (first measurement traits were plant height (H), fresh mass (FM), root length (RL), and the number of leaves (NL) and shoots (NS) per plant). The second measurement was performed after 8 weeks of growth under experimental treatments as follows: low and high light/three planting densities (second measurement traits: plant height (H), leaf length (LL), leaf width (LW), and the number of leaves (NL), internodes (NI), and shoots (NS) per plant). In perennial species, we performed the third measurement after 15 weeks of growth and the traits measured were the same as in the second measurement (the life cycle of *L. purpureum* is up to 15 weeks).

Fresh mass (FM) was measured on a Chyo Japan JL-200 high precision balance that measures mass to the nearest 0.1 mg. For plant size assessment (H, RL, LL, LW), a “Time” digital caliper with 0.01 mm accuracy was used. One focal plant per pot was chosen in each density treatment: the center plant at the highest density (5 plants per pot) and one of three plants was chosen at random in the medium density treatment. The leaf on the second node from the top of the main shoot was measured.

Statistical analyses

Nonparametric Spearman’s rank-order correlations (ρ) between pairs of traits [27] using Statistica software [28] were calculated. To estimate the variability

of correlations, we tested the equality of pairs of correlation coefficients. This was done after the conversion of correlation coefficients using Fisher's Z transformation by conducting a z test [29,30]:

$$Z = z_1 - z_2 / \text{square root of } [(1/N_1 - 3) + (1/N_2 - 3)],$$

where z_1 and z_2 are z values that correspond to correlation coefficients, and N_1 and N_2 are sample sizes. Significant differences in correlation between pairs of traits in either ontogenetic stage, habitat, population, species or treatment indicate that the correlation between characters was altered by any of these factors.

To estimate the level of phenotypic integration, principal component analysis (PCA) and calculation of the index of integration (I) as a variance of the eigenvalues of a correlation matrix [31,32] were performed for every ontogenetic stage, habitat, population, species and treatment. The eigenvalue variance, i.e. the index of integration, scales linearly with the mean correlation square and corresponds better to the overall morphological integration [33,34].

To visualize the patterns of correlations, all trait correlations with values above 0.16 (the smallest value of a significant Spearman correlation) are presented in correlation diagrams (networks) [6,35,36].

RESULTS

Differences in trait correlations among three *Lamium* species

First measurement

During transplantation, the highest level of phenotypic integration ($I=2.19$), without low correlation coefficients ($P<0.3$), was detected in *L. purpureum*. Phenotypic integrations in *L. album* and *L. maculatum* were more than two-fold lower ($I=1.03$ and $I=0.88$ in *L. album* and *L. maculatum*, respectively) (Fig. 1). All correlations that differed significantly ($P<0.05$) between *L. purpureum* and the other two species were higher in *L. purpureum* (Supplementary Table S1).

In later measurements, after 8 and 15 weeks of growth, almost all correlations showed significantly higher values in comparison to the first measurement (Supplementary Table S1). Mean phenotypic integration in perennials was on average 2.6-fold higher in the second and third measurements in comparison to the first measurement (*L. album*: $I_{\text{mean}}=2.65$ and $I_{\text{mean}}=2.12$ in the second and third measurement vs. $I=1.03$ in the first measurement; *L. maculatum*: $I_{\text{mean}}=2.35$ and $I_{\text{mean}}=2.56$ in the second and third measurement vs. $I=0.88$ in the first measurement). In annual *L. purpureum*, the mean phenotypic integration was 1.2-fold higher in the second ($I_{\text{mean}}=2.66$) in comparison to the first ($I=2.19$) measurement (Figs. 1, 2A-C, 3A and B).

Second measurement

After 8 weeks of growth, *L. album* and *L. purpureum* displayed greater correlation coefficients than *L. maculatum* in almost all comparisons (Supplementary Table S1). In all three species, stronger integration was detected in the low light treatment (Fig. 2A-C).

In *L. album*, the highest phenotypic integration ($I=3.26$) was detected in plants grown in the low light treatment and at a density of 3 plants/pot (Fig. 2A). Variability of individual correlations was detected only in the high light treatment. Plants with the lowest integration also had significantly lower values of correlations in comparison to plants from 1 plant/pot density (Supplementary Table S1).

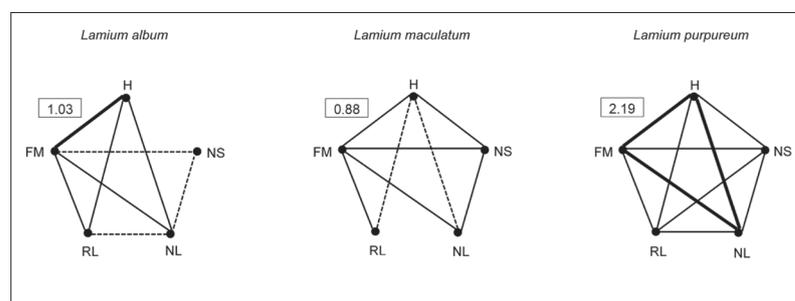


Fig. 1 Correlation networks among traits of *L. album*, *L. maculatum* and *L. purpureum* measured after transplantation, with the levels of phenotypic integration (I-values are in rectangles). Line thickness indicates the magnitude of correlations: thick line $0.7 < P < 1$, medium line $0.3 < P < 0.7$, and dashed line $0 < P < 0.3$. Abbreviations: H, plant height; FM, fresh mass; RL, root length; NL, number of leaves per plant; NS, number of shoots per plant.

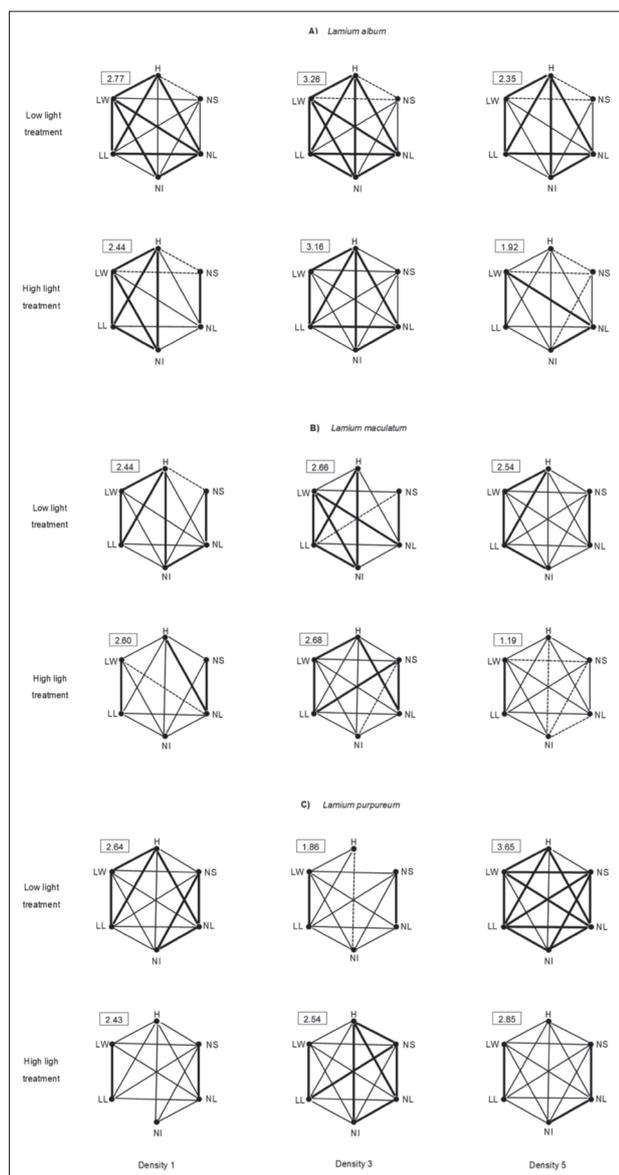


Fig. 2. Correlation networks among traits of *L. album* (A), *L. maculatum* (B) and *L. purpureum* (C) measured after 8 weeks of growth under two light treatments (low and high) and three density treatments (1, 3 and 5 plants/pot), with the levels of phenotypic integration (I-values are in rectangles). Abbreviations: H, plant height; LL, leaf length; LW, leaf width; NL, number of leaves per plant; NI, number of internodes per plant; NS, number of shoots per plant.

The highest integration ($I=2.68$) in *L. maculatum* was detected in the high light treatment with a density of 3 plants/pot (Fig. 2B). Variability in individual correlations was detected also in the high light treatment and between light treatments. Correlations in plants with the highest integration were significantly

higher than those in plants from both 1 plant/pot and 5 plants/pot densities. Plants from the 3 plants/pot density had a greater correlation value in the high light treatment (Supplementary Table S1).

In *L. purpureum*, the highest level of integration ($I=3.65$), the greatest number (12) of correlation coefficients with high values ($P>0.7$) were detected in plants grown in the low light treatment and the highest density (Fig. 2C). Variability in individual correlations was detected in both light treatments, as well as between them. In the low light treatment, correlations in the 3 plants/pot density were significantly lower than in both other densities, and also lower than in the high light treatment. In the high light treatment, significantly greater correlations were detected in the density of 5 plants/pot in comparison to the 1 plant/pot density (Supplementary Table S1).

A strong correlation was detected between leaf width and leaf length ($P>0.7$) in all light/density treatments in both *L. album* and *L. maculatum* (Fig. 2A-C). In *L. purpureum*, two correlations had high values throughout all treatments, between leaf length and leaf width, and between the number of shoots and the number of leaves (Fig. 2C).

Third measurement

Fifteen weeks after transplantation, stronger integration was detected in the high light treatment in both perennial species (Fig. 3A and B). In *L. album*, the highest integration ($I=3.40$) and the greatest number (12) of correlation coefficients with high values were detected in the high light treatment and the highest density (Fig. 3A). In *L. maculatum*, the highest integration ($I=3.43$), the greatest number (11) of correlation coefficients that had high values and without low correlation coefficients, were detected in plants grown under the high light treatment and at the lowest density (Fig. 3B). Variability in individual correlations was observed in both light treatments, as well as between light treatments in both species (Supplementary Table S1).

Three pairs of traits in *L. album* showed a strong correlation ($P>0.7$) in all treatments: leaf width and leaf length, the number of shoots and the number of leaves, height and the number of internodes (Fig. 3A).

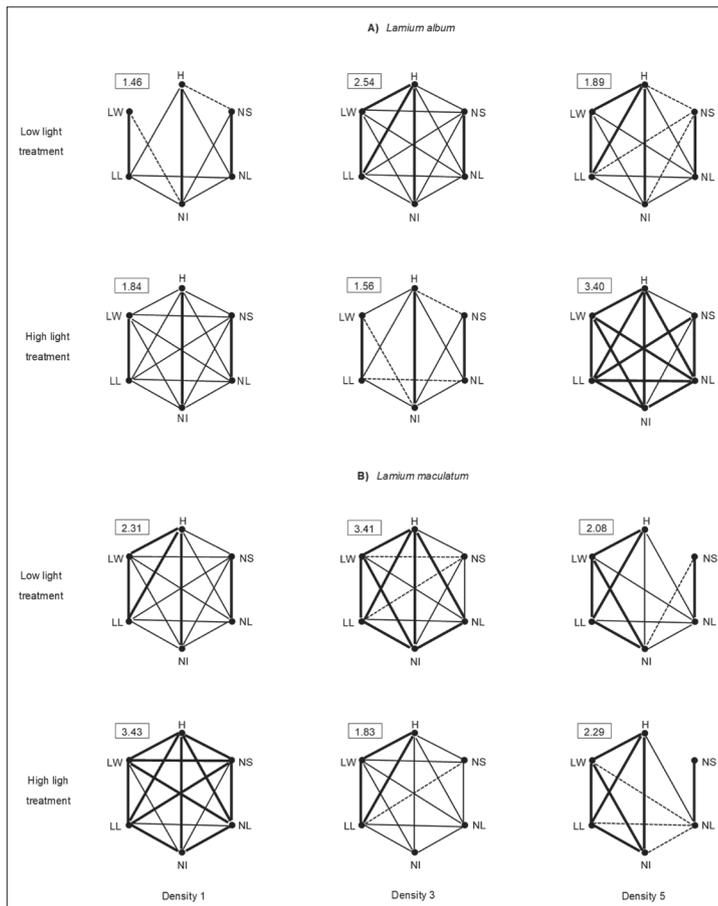


Fig. 3. Correlation networks among traits of *L. album* (A) and *L. maculatum* (B) measured after 15 weeks of growth under two light treatments (low and high) and three density treatments (1, 3 and 5 plants/pot), with the levels of phenotypic integration (I-values are in rectangles). For abbreviations see Fig. 2.

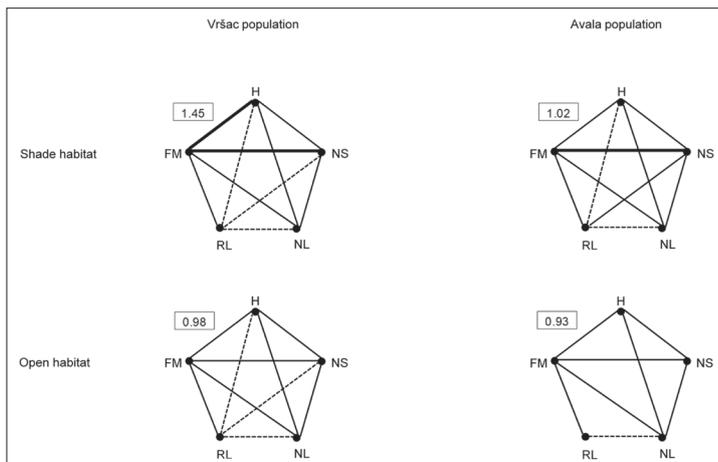


Fig. 4. Correlation networks among traits of *L. purpureum* from the Vršac and Avala populations and two habitats (shade and open) measured after transplantation with the levels of phenotypic integration (I-values are in rectangles). For abbreviations see Fig. 1.

In *L. maculatum*, three pairs of traits also had high values in all treatments: leaf width and leaf length, height and leaf width, and height and leaf length (Fig. 3B).

Differences in trait correlations between populations and habitats of *L. purpureum*

First measurement

During transplantation, shade habitats showed higher integration than open habitats in both populations. The highest integration ($I=1.45$) was detected in plants that originated from the Vršac population (Fig. 4). All correlations that differed significantly between habitats within both populations were also higher in the shade than in the open habitat (Supplementary Table S2).

Second measurement

After 8 weeks of growth, almost all correlations showed significantly higher values in comparison to the first measurement (Supplementary Table S2). Mean phenotypic integration was on average 1.9-fold higher in the second measurement in comparison to the first measurement (Vršac population: $I_{\text{mean}}=2.02$ in the second measurement vs. $I=1.22$ in the first measurement; Avala population: $I_{\text{mean}}=2.08$ in the second measurement vs. $I=0.98$ in the first measurement) (Figs. 4 and 5A and B).

Plants that originated from the Vršac population and the open habitat showed generally higher integration in comparison to those in the shaded habitat. Plants that originated from the open habitat had the highest integration ($I=2.35$) in the high density treatment (Fig. 5A). Variability between the density treatments was detected only in plants that originated from the shaded habitat (3 correlations were significantly greater in the low in comparison to high density) (Supplementary Table S2).

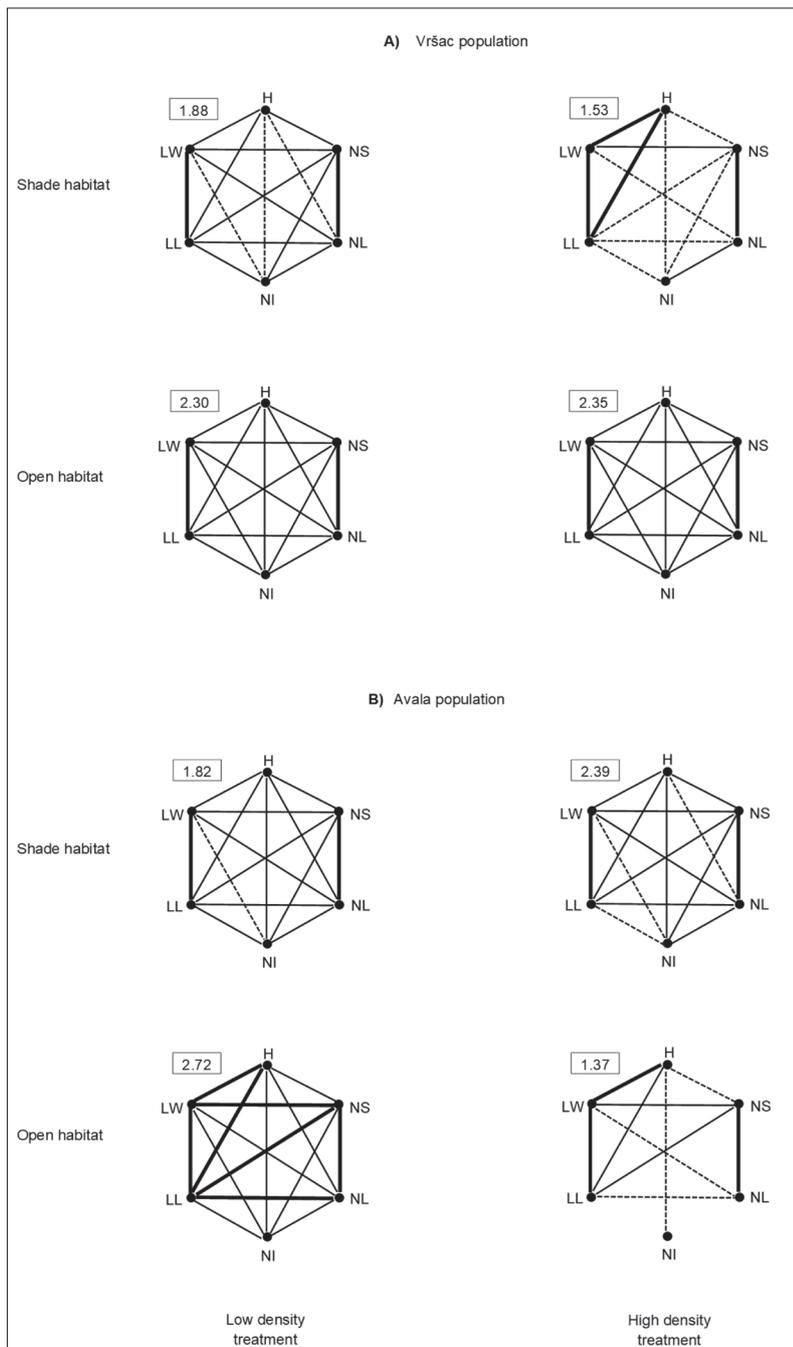


Fig. 5. Correlation networks among traits of *L. purpureum* from Vršac (A) and Avala (B) populations and two habitats (shade and open) measured after 8 weeks of growth under two density treatments (low and high) with the levels of phenotypic integration (I- values are in rectangles). For abbreviations see Fig. 2.

Plants that originated from Avala showed the highest integration in the open habitat under the low density treatment ($I=2.72$) (Fig. 5B). Variability between density treatments was detected only in plants

that originated from the open habitat (11 correlations were significantly greater in the low density in comparison to the high density) (Supplementary Table S2).

In both Vršac and Avala populations, correlations between leaf width and leaf length and between the number of shoots and the number of leaves showed strong correlations ($P>0.7$) in all habitat/light treatments (Fig. 5A and B).

Differences between the Vršac and Avala populations were detected mainly in the high density treatment. Vršac had higher correlation values than Avala in the open habitat, and Avala had higher correlation values than Vršac in the shaded habitat (Supplementary Table S2).

DISCUSSION

The strength and pattern of phenotypic correlations in *L. purpureum* were more distinctive in comparison to the other two species exposed to uniform growing conditions, i.e. during transplantation. The highest integration and the strongest trait correlations were identified in *L. purpureum* in comparison to *L. album* and especially to *L. maculatum*. After 8 weeks of growth, only *L. purpureum* showed variability in response to density in both light treatments, as well as the strongest integration in the potentially most stressful environment. The heterogeneous environments (open habitats) that *L. purpureum* inhabits favor the evolution of phenotypic plasticity. Variability of correlations usually occurs as a consequence of differential plastic responses of individual traits [37,38]. Because plastic responses must be coordinated, the evolution of flexible phenotypic correlation structure and phenotypic integration are

also favored [38]. Low light intensity and the highest planting density are potentially the most stressful combination for this species [26]. The strongest integration detected in *L. purpureum* confirms the hypothesis that integration should be higher under environmental stress [16]. Moreover, our previous phenotypic selection analysis showed that increased density also led to reduced fitness [24]. An increase in trait integration with soil metal toxicity was also found in 65 representative species of copper flora from the Katangan Copperbelt [39]. The opposite was found for *Arabidopsis thaliana* in competition with *Lolium multiflorum* [40].

Responses of *L. album* and *L. maculatum* to the applied treatments changed in the later stages of development, after 15 weeks of growth. Both species showed variability in response to density in both light treatments, as well as between treatments, as was detected for *L. purpureum* after 8 weeks of growth. In addition, the patterns of correlations in plants with the highest integrations resembled that in *L. purpureum* after 8 weeks. This response indicated slower development and delayed reaction to treatments in perennial species [41,42]. A reason for the delayed reaction could be the simultaneous effect of multiple stresses, when two or more stresses co-occur, one of which could have priority. A lower reaction to density in perennials could be the consequence of stress caused by shade, which along with the small plant sizes could not create enough competition for aboveground and belowground resources [24,43]. It was observed that biomass allocation to roots in perennials is higher and increases more strongly in response to density than in annuals [44]. A lower correlation variability in response to density mirrored the lower variability in trait values and plasticity along the density gradient, as detected in previous analyses [24].

The highest integration in *L. maculatum* was found at the lowest density, and the highest integration in *L. album* was found at the highest density, both under the high light treatment. In the perennial species we did not find a potentially stressful environment to be a straightforward predictor of the level of phenotypic integration, which could be a consequence of their clonality [26]. *L. album* is rhizomatous while *L. maculatum* is a stoloniferous species, and both efficiently cope with environmental heterogeneity. Consequently, when subjected to heterogeneous environ-

mental conditions, they buffer environmental stresses by inhabiting favorable patches and by physiological integration where donor ramets help others to resist stress and disturbances [45]. A lack of a higher level of integration in some potentially stressful treatments was also reported in a study on *Arabidopsis thaliana* subjected to different water and light levels [46].

It was proposed that phenotypic correlations differ more between less phylogenetically related species, but this is not in agreement with the results of our experiment [38,47]. *L. maculatum* and *L. purpureum* are phylogenetically closer to each other than to *L. album*. Therefore, differences in phenotypic correlations among species are more related to interspecific niche differences (ecological and phenological ones) than to phylogenetic distances [48].

In the additional experiment performed on *L. purpureum*, we detected stronger integration during transplantation in plants that originated from shaded habitats. This could be the consequence of light deprivation as a potential stress for *L. purpureum*, which generally prefers unshaded environments [16,26].

In the later stages of development (after 8 weeks), higher integration was detected under the low density treatment in both populations. This kind of similarity in morphological integration was also found in two populations of *Phlox drummondii* grown in three different nutrient environments [38]. On the other hand, only plants that originated from the Vršac shaded and Avala open habitats exhibited variability in response to density treatments. This is in agreement with the findings from a previous study [25] where subpopulations occupying different habitats differed in their plastic responses to density, and where between-habitat differences were also population-specific. In [25], greater plasticity was detected also in the Avala population. Thus, both greater plasticity and correlation variability could be caused by a higher R:FR, and therefore greater sensitivity to R:FR change in the Avala habitats.

Like the results of the first experiment on the three *Lamium* species, there was also a greater difference in response to density treatments between habitats than between populations of *L. purpureum*. Therefore, correlations are shaped more by the environment than by interpopulation distances. Correspondingly, floral morphology in populations of *L. album* var. *barba-*

tum varied in relation to local pollinator size and did not reflect the degree of genetic relatedness among populations [49].

The lower integration levels and lower values of correlation coefficients detected in juvenile plants of all three species in both experiments could enable a flexible response to the environment in which they germinate and develop. Integration changes during ontogeny and phenotypes become more integrated in later stages of development [7,47]. The opposite was found for the rhizomatous monocot *Iris pumila*, but after a much longer growth period [5].

In both experiments, the correlation between leaf length and leaf width always had high values. This correlation is often found in published data, as for example in populations of *Phlox drummondii* grown in different nutrient environments [38], as well as in the majority of hybrid and parental species in the family Brassicaceae [50]. *Turnera velutina* families with higher phenotypic integration among leaf functional traits had faster growth and produced more flowers [51]. In *L. purpureum* plants, the correlation between the number of shoots and the number of leaves was also high. All these traits exhibited similar reactions in response to applied treatments [24,25]. These results suggest that characters that have similar reaction patterns are more firmly correlated than those that do not [52,53].

We can conclude that *L. purpureum* showed more flexible phenotypic correlations in response to the applied treatments than the perennials, *L. album* and *L. maculatum*. Variability in phenotypic response in perennials developed more slowly, intensified over time and became similar to that of *L. purpureum*. The differences in phenotypic correlations among species, as well as between *L. purpureum* populations, correlated more with ecological than with phylogenetic differences.

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Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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Supplementary Material

The Supplementary Material is available at:
Supplementary Table S1. http://www.serbiosoc.org.rs/NewUploads/Uploads/Barisic%20Klisaric_6596_Supplementary%20Material.Fig.%20S1.xlsx
Supplementary Table S2. http://www.serbiosoc.org.rs/NewUploads/Uploads/Barisic%20Klisaric_6596_Supplementary%20Material.Fig.%20S2.xlsx