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Reply to: Differences between dumbbell and kidney-bean stomatal types may influence relationships between stomatal traits and the environment

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REPLYING TO K. Xing et al. *Nature Communications* https://doi.org/10.1038/s41467-025-61635-y (2025)

Stomata are the valves controlling the exchange of carbon dioxide and water vapor between plants and the atmosphere, and their evolution played a crucial role in enabling the colonization of terrestrial habitats and formation of terrestrial ecosystems[1,2]. Stomata are made up of pairs of guard cells, which are kidney-shaped in most plants, and dumbbell-shaped in grasses, and flanked by specialized subsidiary cells. Several studies among small groups of diverse species showed that opening and closing processes of these stomatal types differ. The elongated dumbbell shaped guard cells of grasses results in a rectangular pore that can change in area with minimal changes in stomatal width[3]. In general, dumbbell shaped guard cells have lower volume than kidney shaped guard cells, and thus higher membrane surface area relative to volume, which would contribute to greater rates of ion transport between subsidiary and guard cells and less requirement for solutes and water for changes in turgor, resulting in more rapid changes in volume for opening and closing[4,5]. The specialized subsidiary cells of grasses have also been implicated in rapid changes in guard cell turgor and stomatal apertures[3,6–8]. The more rapid stomatal responses of dumbbell shaped guard cells may also contribute to greater water-use efficiency under fluctuating light[5]. Yet, the importance of guard and subsidiary cell anatomy on stomatal responses and their relevance to whole plant function are not completely understood. Studies of more diverse species as well as greater numbers of species within the grass lineage are required to answer these questions, and will be critical for applying grass leaf physiological processes to ecological prediction and agricultural productivity.

Xing et al.[9] emphasize the importance of stomata in ecosystem function, and the potential need to consider these distinct stomatal types, and how they might have contrasting influence on patterns of ecosystem adaptation across climatic gradients. In particular, Xing et al.[9] raised the question of whether the existence of two stomatal types within ecosystems should preclude considering combining species with the different stomatal types in the calculation of community mean trait values and in determining how these vary across environmental gradients. Specifically, Xing et al.[9] argued that the meaningfulness of the community-weighted mean value for stomatal length (SL) and its relationship to climate at the continental scale hinges on whether the SL of the two stomatal types exhibit similar responses to the environment. This line of inquiry presents the opportunity to consider key questions about stomatal adaptation, i.e., do grasses and non-grass stomatal traits vary with environment similarly. Further, the questions of Xing et al.[9] point to a potential importance of considering "community lineage means" for different functional groups or lineages, in addition to the all-species community weighted means typically used by ecologists to compare ecosystems. Here we address these questions with new analyses of our dataset from Liu et al.[10] (Supplementary Data 1), providing insights both about stomatal variation and ecological trait patterning with respect to the environment.

Results and Discussion

In our first analysis, as suggested by Xing et al.[9], we address the potential differences between the two ecosystem types—grasslands

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and forests—in their association of stomatal traits with environment. That question was beyond the scope of our original paper, but we agree with Xing et al.[9] that it is a highly relevant one, and thus we test for contrasting stomatal trait-environment relationships that may be linked with the different guard cell shapes of their dominant species. Our detailed analysis shows some differences in the environmental associations of stomatal traits for forest and grassland communities using community weighted metrics, though perhaps surprisingly, most environmental associations are similar for the two ecosystem types. When we use a principal components analysis of 30 environmental variables (including, e.g., mean annual temperature and precipitation and many others; Table S1), the PC axes 1, 2 and 3 explain 82.1% of the total variation (Table S2). We analyze environmental trends for community-weighted means of SL and stomatal density (SL$_{\text{CWM}}$ and SD$_{\text{CWM}}$, respectively) with these PCA axes and with individual climate variables (Tables S3, 4). Across forests, the SL$_{\text{CWM}}$ increases strongly with climate PC1 ($R^2 = 0.32$, $t = 3.94$, $df = 26$, $p = 0.001$), corresponding to a negative trend with moister, warmer climates, whereas grasslands do not show a significant association ($R^2 = 0.009$, $t = -0.58$, $df = 27$, $p = 0.56$). However, forests and grasslands show statistically similar slopes for their responses of SL$_{\text{CWM}}$ to climate PC2 and PC3, and differ only in intercepts, consistent with the larger SL of grasslands than forests (Fig. 1). Further, the forests and grasslands do not differ in the slopes of their responses of SD$_{\text{CWM}}$ to climate PC1, PC2 and PC3 (Fig. 1). Tests of the responses of SL$_{\text{CWM}}$ and SD$_{\text{CWM}}$ to individual environmental variables are consistent; respectively, 19/30 (63.3%) and 28/30 (93.3%) of their associations with environment variables have similar slopes for grasslands and forests (Tables S3, 4). For six of the 11 SL$_{\text{CWM}}$-environment associations that differ significantly in slope, forest ecosystems show a significant association with environment and the grasslands do not. The possible reason for the greater responses of SL$_{\text{CWM}}$ of forests than grasslands

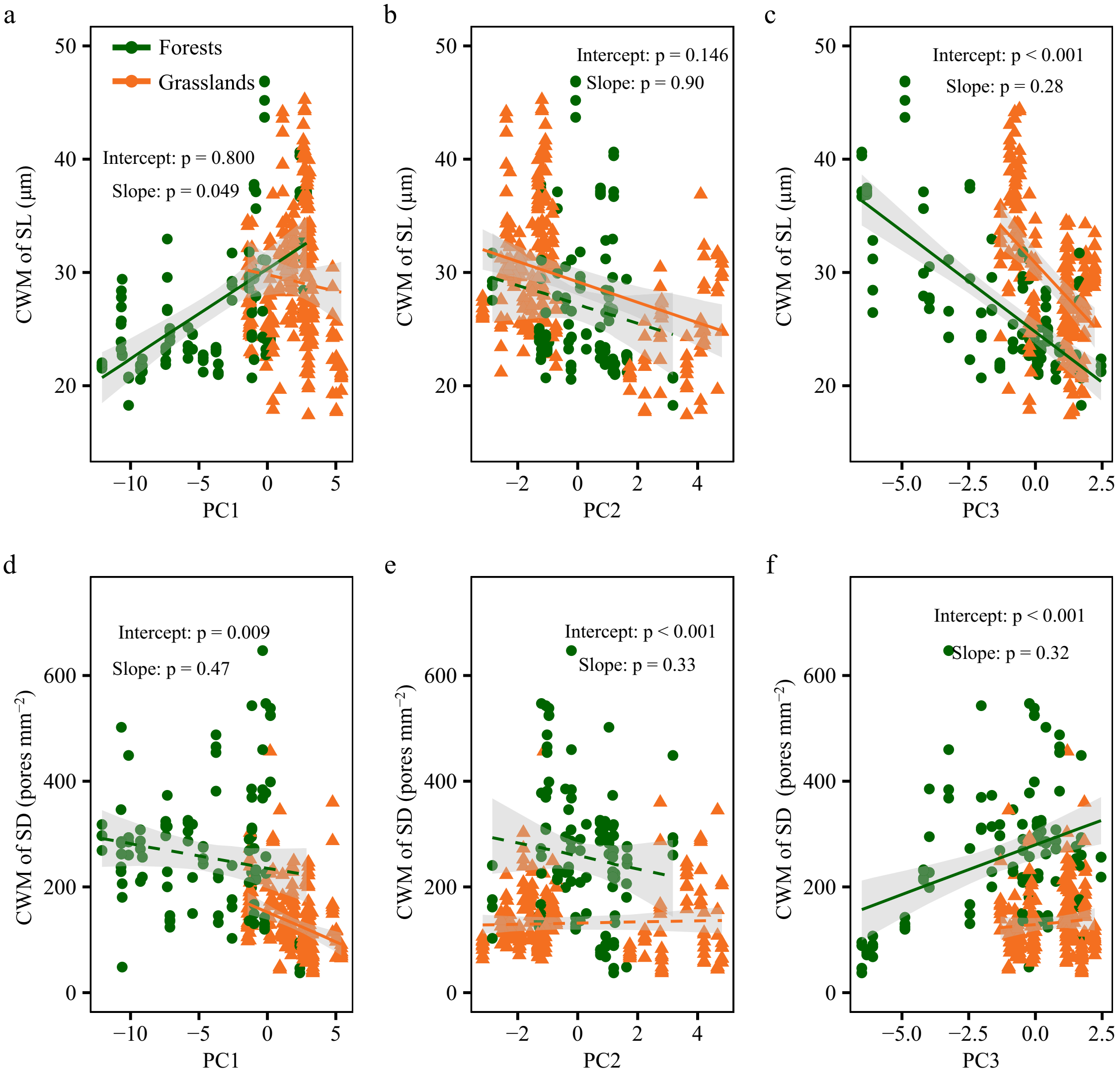

Fig. 1 | Relationships of community-weighted means of stomatal traits across 28 forests and 29 grasslands to the environment. CWM community-weighted mean, SL stomatal length, SD stomatal density, PC1, PC2, and PC3 represent the first three axes of the Principal Component Analysis (PCA) for 30 environmental variables, respectively. **a–c** Relationships of CWMs of SL to PC1, PC2, and PC3, respectively. **d–f** Relationships of CWMs of SD to PC1, PC2, and PC3, respectively. Statistical analysis was performed using linear mixed-effects models, with vegetation type (forests *vs.* grasslands), environment, and their interaction as fixed effects, and plot nested within site as a random effect. The solid lines depict significant linear regressions and the dashed lines non-significant linear regressions, and the gray shading indicates 95% confidence intervals. We included the regression lines for both significant and non-significant relationships to allow for direct visual comparison of slopes and intercepts between the two groups. The *p*-value of < 0.05 signifies significant differences in the slopes and intercepts of the relationships of stomatal traits for forests and grasslands to the environment. For additional details, please refer to Tables S3, 4. Source data are provided as a Source Data file.

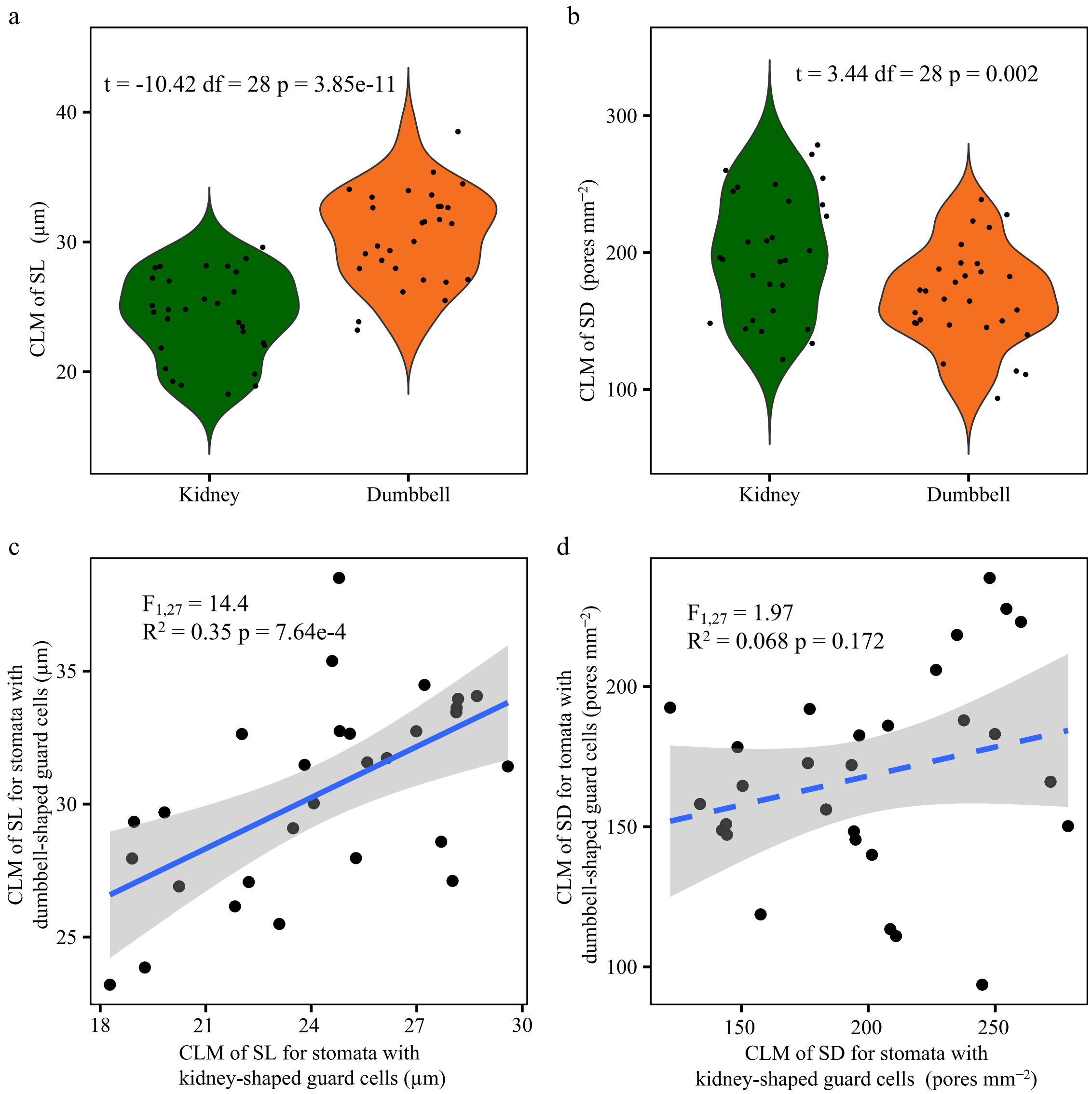

Fig. 2 | Changes in community lineage means of stomatal traits for stomata with dumbbell versus kidney-shaped guard cells and their relationships across 29 grassland sites. CLM community lineage mean, SL stomatal length, SD stomatal density. **a**, **b** Differences in CLM of SL and SD between plants with dumbbell-shaped and kidney-shaped guard cell stomatal types, respectively. **c**, **d** Relationships of CLM of SL and SD between plants with dumbbell-shaped and kidney-shaped guard cell stomatal types across 29 grassland sites, respectively. Violin plots of stomatal traits for dumbbell-shaped and kidney-shaped guard cell stomatal types are filled with different colors. Two-sided paired sample t-tests were employed to investigate differences in stomatal traits between stomatal types. Ordinary least squares regression was used to explore relationship between the CLM of stomatal traits for dumbbell-type and kidney-type stomata. The solid and dashed lines depict significant and non-significant linear regressions, respectively, while the gray shading indicates the 95% confidence interval. We included the regression lines for both significant and non-significant relationships to allow for direct visual comparison of slopes and intercepts between the two groups. Source data are provided as a Source Data file.

might relate to differences in leaf or stomatal type of the component species, with a greater advantage for relatively smaller stomata in colder and drier conditions on average for forest species. Alternatively, the stronger trends for forests than grasslands may be due to the larger range of climates occupied by forests than grasslands in our study.

Yet, that analysis, as suggested by Xing et al.[9], does not directly address their question of whether grasses and non-grasses, with their different stomatal types, differ in their environmental associations across communities. Indeed, forests and grasslands both include a mixture of many lineage groups, and grasslands contain both grasses and non-grass species. Therefore, for a more appropriate analysis of the differences between species of contrasting stomatal types in their climate associations, for the 29 grassland sites we calculate “community lineage mean” values of SL and SD (SL_{CLM} and SD_{CLM}, respectively), representing the grasses (dumb-bell shaped guard cells) and non-grasses (kidney bean shaped guard cells) and test their associations with environment across sites. Notably, across these 29 grassland sites, SL_{CLM} is higher and SD_{CLM} lower for grasses than non-grasses (Fig. 2), and grasses and non-grasses are positively related in their SL_{CLM} ($R^2 = 0.35$, $p < 0.001$, $F_{1,27} = 14.39$), and independent in their SD_{CLM} ($R^2 = 0.068$, $p = 0.17$, $F_{1,27} = 1.97$; Fig. 2). When we use a principal components analysis of 30 environmental variables across 29 grassland sites, the PC axes 1, 2 and 3 explain 85.2% of the total variation (Table S5). In our analyses of trends for SL_{CLM} and SD_{CLM} for grasses and non-grasses with environmental variables, the SL_{CLM} for grasses and non-grasses show similar relationships with environment, with no differences in slope with PC1, PC2 or PC3, whereas SD_{CLM} for grasses and non-grasses show different slopes for PC1 but not PC2 and PC3

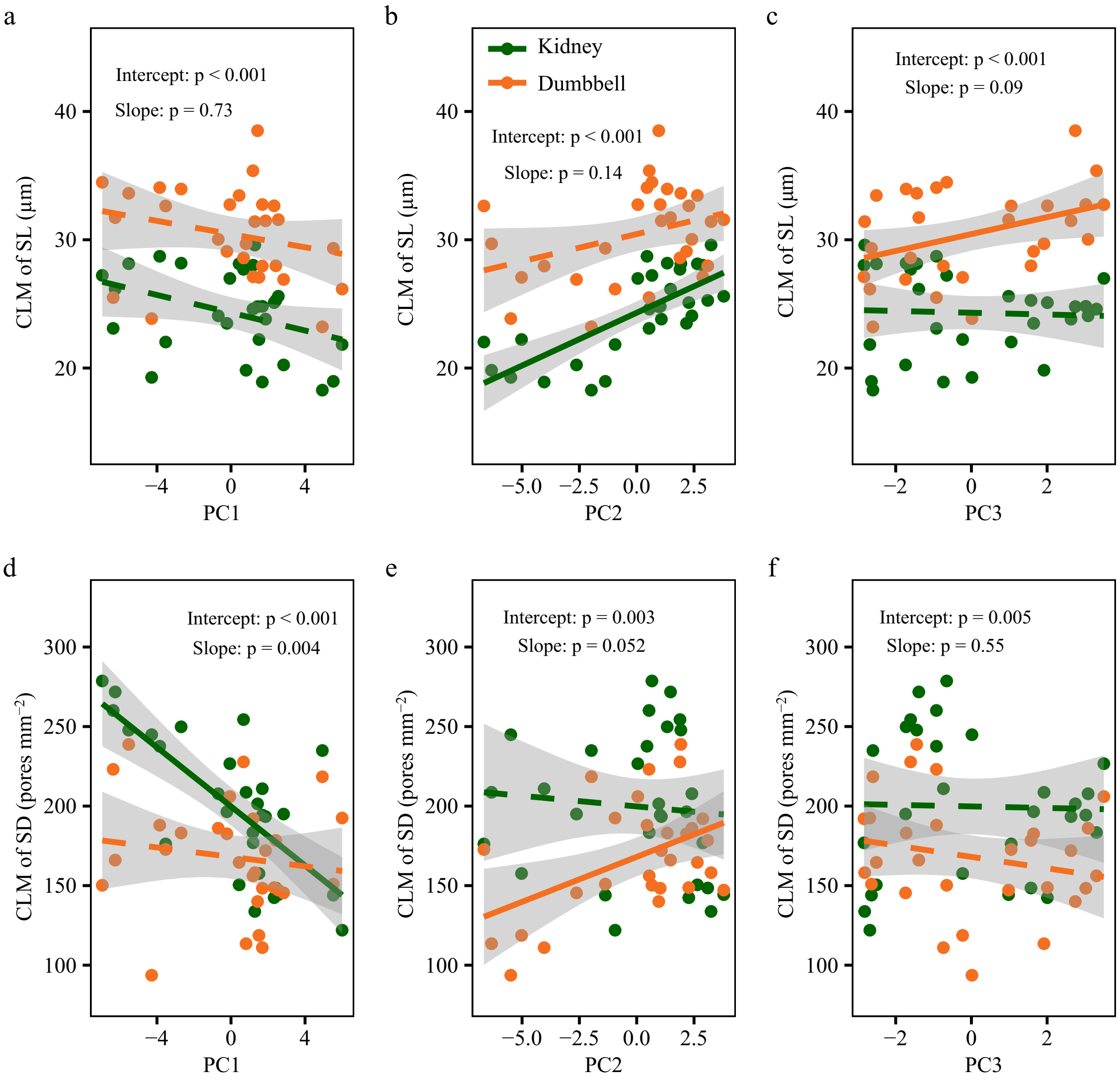

Fig. 3 | Relationships of the community lineage means of stomatal traits for grasses versus non-grasses (with stomata with dumbbell- versus kidney-shaped guard cells, respectively) to the environment across 29 grassland sites. CLM community lineage mean, SL stomatal length, SD stomatal density. PC1, PC2, and PC3 represent the first three axes of the Principal Component Analysis (PCA) for 30 environmental variables, respectively. **a**–**c** Relationships of CLM of SL to PC1, PC2, and PC3, respectively. **d**–**f** Relationships of CLM of SD to PC1, PC2, and PC3, respectively. Ordinary least squares regression was used to explore relationship between the CLM of stomatal traits and environment. The differences in regression slopes and intercepts were assessed using analysis of covariance (ANCOVA). The solid lines depict significant linear regressions and the dashed lines non-significant linear regression; the gray shading indicates 95% confidence intervals. We included the regression lines for both significant and non-significant relationships to allow for direct visual comparison of slopes and intercepts between the two groups. The *p*-value of <0.05 signifies significant differences in the slopes and intercepts of the relationships of stomatal traits for dumbbell-shaped and kidney-shaped guard cell stomatal types to the environment. For additional details, please refer to Table S6, 7. Source data are provided as a Source Data file.

(Fig. 3). Tests of the responses of SL_{CLM} and SD_{CLM} to individual environmental variables are consistent. For SL_{CLM}, 30/30 (100%) of associations have similar slopes for grasses and non-grasses (Tables S6, 7). For SD_{CLM}, 19/30 (63.3%) of associations have similar slopes for grasses and non-grasses (Tables S6, 7); for one of the 11 SD_{CLM}-environment associations that differs significantly in slope for grasses and non-grasses, the grasses show a significant association with environment and the non-grasses do not, and for 9 the non-grasses show a significant association with environment and the grasses do not. We conclude that the observed differences in SL_{CWM}-environment relationships between forests and grasslands (Fig. 1) are not likely due to the differences between grasses and non-grasses in their stomatal types, as the responses of SL to the environment are almost identical for community lineage means representing the two stomatal types in grassland species.

We conduct a third analysis to consider one of our original study's major findings—the stomatal length-density trade-off across communities at continental scale[10]—and whether this relationship may vary for community weighted means for grasslands versus forests, or for community lineage means for grasses versus non-grass species of grasslands. The trade-off between stomatal length and density is robust in all tests for forests and grasslands, and for grasses and non-grasses (Fig. 4). The slope and intercept of the SL_{CWM} versus SD_{CWM} regression are both higher for forests than grasslands, but the slopes and intercepts are statistically similar for SL_{CLM} versus SD_{CLM} for grasses and non-grasses across the 29 grassland sites. As in the previous analysis, we conclude that stomatal trait versus environment relationships do differ between forests and grasslands, but likely not due to the differences between grasses and non-grasses in their stomatal types.

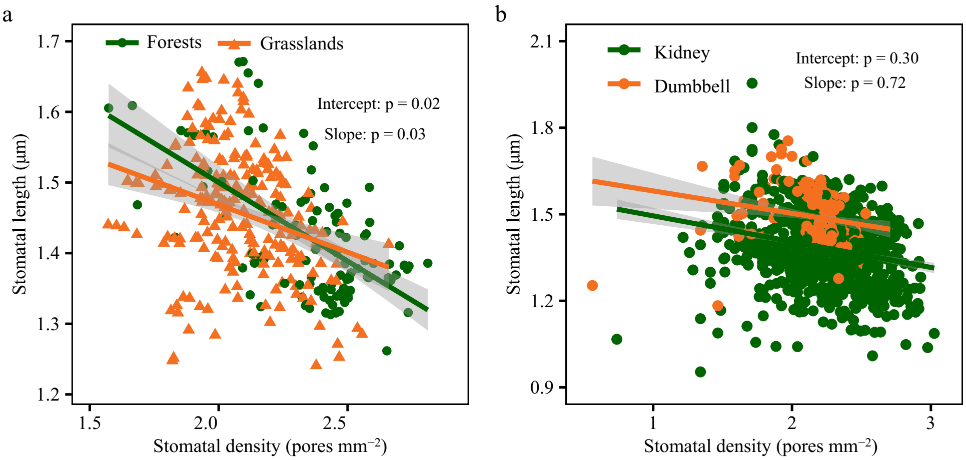

Fig. 4 | Trade-off between stomatal density and length. a Trade-off between community-weighted means of stomatal density and length across 28 forest sites and 29 grassland sites. **b** Trade-off between stomatal density and length across grasses (with dumbbell shaped guard cells) and non-grass (with kidney-bean shaped guard cells) species. All stomatal traits are log-transformed. Ordinary least squares regression was used to explore relationship between stomatal density and length. The solid lines depict significant linear regressions, and the gray shading indicates the 95% confidence intervals. The differences in regression slopes and intercepts were assessed using analysis of covariance (ANCOVA). The *p*-value of <0.05 signifies significant differences in the slopes and intercepts of the linear regression between stomatal density and length. Source data are provided as a Source Data file.

Overall, our results suggest that the majority of environmental associations of SL and SD are consistent between grasslands and forests, considering their community weighted means, and between grasses and non-grasses, considering their community lineage means. These findings are consistent with the stomatal types (dumbbell-shaped and kidney-shaped guard cells) exhibiting similar adaptation to environmental factors at the scale of communities across the continent. That convergence may be explained by the vast differences in timescale between stomatal dynamic movements (minute to second scale) and community assembly and environmental adaptation across a continent (many years to millennia). Thus, while dumbbell-shaped and kidney-shaped stomata would exhibit differences in speed of dynamic opening and closing, our results suggest that their long-term adaptations tend to be similar given the many other processes that would influence adaptation across larger scales of space and time.

Finally, we wish to address the conclusions of Xing et al.[9] that "principles of consistency" should prohibit testing the associations of community weighted means with climate when communities are comprised of diverse lineage groups with strong differences in physiology. We found broadly similar patterns when comparing community weighted means and community lineage means for the adaptation of stomatal traits to the environment at continental scale. Recent studies have emphasized that for inputting traits to represent ecosystems in vegetation models, using values averaged for species within lineages can provide improved resolution over values averaged for species within coarse categories of plant functional types based on growth form, phenology, photosynthetic pathway and climate (e.g., temperate vs tropical)[11,12]. Yet, we argue that when trait data are available for a majority of plant species, or the dominant species of communities, community weighted means remain highly informative to represent ecosystems[13], and thereby to address many questions in evolution, community assembly and global change biology, including ecosystem responses to climate.

Methods

The detailed procedures for study site selection, field sampling, and stomatal measurements are documented in the study of Liu et al.[10].

In our previous study, we selected 30 environmental variables to examine the relationships between stomatal traits and the environment (Table S1). Here, principal component analyses (PCA) were conducted on these environmental variables across all forest and grassland sites. The first three axes of the PCA (PC1, PC2, and PC3) collectively explained 82.1% of the variation in all environmental variables. To test the differences in stomatal trait-environment relationships for forests and grasslands, these 30 environmental variables, along with PC1, PC2, and PC3 (serving as proxies for the 30 environmental variables), were respectively included in the Analysis of Covariance (ANCOVA) models. The models were written as: stomatal trait ~ environment * vegetation + (1|site). Significance of the interaction term between environment and vegetation type in the regression model would indicate that the stomatal trait-environment relationships were different for forests and grasslands.

Mean stomatal traits values of the two stomatal types (dumbbell-shaped and kidney-shaped guard cells) were separately calculated for each grassland site. Paired sample t-test was employed to investigate differences in SL_{CLM} and SD_{CLM} between dumbbell-shaped and kidney-shaped guard cell types across 29 grassland sites, and their relationship was tested using linear regression. At the site level, we explored stomatal trait-environment relationships for both stomatal types. To test the differences in stomatal trait-environment relationships for the stomatal types, ANCOVA was also employed. Principal component analyses (PCA) were performed on 30 environmental variables across all grassland sites, the first three axes of the PCA (PC1, PC2, and PC3) collectively explained 85.2% of the variation in all environmental variables. These 30 environmental variables, along with PC1, PC2, and PC3 (serving as proxies for the 30 environmental variables), were respectively included in the Analysis of Covariance (ANCOVA) models. The models were written as: stomatal trait ~ environment * stomatal type. Significance of the interaction term between environment and stomatal type in the regression model would indicate that the stomatal trait-environment relationships were different for kidney and dumbbell-shaped stomata.

Following similar procedures, we extended our analysis to investigate whether there were differences in the trade-off between stomatal density and length for grasslands versus forests, as well as between kidney and dumbbell-shaped guard cell stomatal types.

All data analyses and figure generation were performed using the R version 4.4.2 (https://www.r-project.org/), with the significance level set to 0.05.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study can be found on Figshare at https://doi.org/10.6084/m9.figshare.28229027.v1. Source data are provided with this paper.

Code availability

The R codes supporting the findings of this study can be accessed on Figshare at https://doi.org/10.6084/m9.figshare.28229027.v1.

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Author contributions

N.H., L.S., C.L., and G.Y. designed the study; C.L., L.S., A.S.B., Y.L., J.Z., and K.Y. drafted the manuscript; L.S., A.S.B., and C.L. revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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