



The effect of local perennial plants on the occurrence and traits of the *Brachypodium distachyon* complex along an aridity gradient

Megan K. Korte¹ · Antonio J. Manzaneda² · Luisa M. Martinez² · Tamatha A. Patterson³ · Rampal S. Etienne¹ · Louis van de Zande¹ · Christian Smit¹

Received: 22 July 2024 / Accepted: 3 March 2025 / Published online: 17 March 2025
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Abstract

Plant-plant interactions are well-known drivers of plant community dynamics and plant traits. Less well known is to what extent these interactions and their effects change along stress gradients. To study this, we assessed the local spatial association of the annual grass species *Brachypodium hybridum* and *Brachypodium distachyon* to perennial plants in ten sites along an aridity gradient in southeastern Spain and compared ten phenotypic traits of *Brachypodium* occurring underneath and outside the perennial plant canopy. We found that *Brachypodium* was less associated with perennials with increasing aridity but with a significant site effect. This site effect seemed to be due to one particular site, the removal of which from our analysis resulted in an even stronger negative correlation and uncovered a significant influence of the perennial plant. We also found that while overall three phenotypic traits (plant height, spike length, and seed number) were different between plants underneath perennial plants and plants outside perennials, only one of these differences (spike length) changed significantly along the gradient. Further, in controlled conditions, we found that the offspring of annual plants collected from outside perennial plant canopies in the least arid site flowered more rapidly than those from underneath perennial plants, but this difference was not observed for the most arid site. These results indicate that in patchy arid landscapes dominated by perennial plants, *Brachypodium*'s response to the microhabitat provided by neighboring perennial plants changes along the aridity gradient, but other factors such as site and perennial plant may counteract or strengthen the effects of aridity.

Keywords *Brachypodium* · Aridity · Local plant interactions · Annuals · Perennials

Introduction

Over 40% of terrestrial environments consist of dryland habitats (Maestre et al. 2021). In the Mediterranean Climate areas (MEA 2005; Sala et al. 2000) climate change is significantly reducing the biodiversity of these semi-arid landscapes. These water-limited habitats, generally

composed of perennial shrubs and annual herbaceous cover, seem especially affected by an increase in temperature and enhanced drought severity (Collins et al. 2014; IPCC 2014). Spatially heterogeneous landscapes, such as the shrub-dominated mountainous landscapes found in southern Spain, are particularly interesting to study the effect of aridity. Although large-scale gradients are commonly used to infer landscape-level patterns (García-Camacho et al. 2017; Manzaneda et al. 2012), various mechanisms driving spatial patterns often actually occur at the smaller microgeographic scale (Richardson et al. 2014) as plants tend to respond to local microclimates (Scherer and Körner 2011). The value of a stress gradient lies within its ability to test a species' response across a range of environmental conditions. Aridity gradients in semi-arid landscapes capture not only the amount of rainfall or the temperature, but also the evaporation and transpiration of water through the landscape (Zomer et al. 2022), and are strong predictors of plant association dynamics

Communicated by Devan McGranahan.

✉ Megan K. Korte
m.k.korte@rug.nl

¹ Groningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

² Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, Jaén, Spain

³ United States Fish and Wildlife Service, Anchorage, Alaska, USA

in drylands (Berdugo et al. 2020). Thus, it is crucial to examine mechanisms governing plant communities at both the macro and microclimate levels, and particularly whether the microscale-level effects change at different macroscales.

It is well known that biotic interactions, both negative and positive, influence the structure of plant communities (Bertness and Callaway 1994; Tilman 1982). Interspecific facilitation is a mechanism by which stress-tolerant perennial plant species positively affect spatially associated beneficiary species (Berdugo et al. 2019; Bruno et al. 2003) and tends to become more prevalent as stressors increase (e.g. Stress Gradient Hypothesis SGH) (Bertness & Callaway 1994). For example, drought-resistant shrubs can alter the local microhabitat by moderating excessive light levels and tempering extreme temperatures (Segoli et al. 2012), increasing soil moisture and nutrients directly beneath their canopies (Armas and Pugnaire 2005; Prieto et al. 2010), providing protection from predation (Smit et al. 2007; Verwijmeren et al. 2013) and affecting soil microorganisms which can promote growth and survival of annual plants (Lozano et al. 2017). As a result, these so called ‘nurse plants’ positively influence the growth, average levels of fitness (Hacker and Gaines 1997), and survival of neighboring plant species (Callaway et al. 2002; Holzapfel et al. 2006). The net positive or negative (competition) effects that neighboring perennial plants can contribute to their beneficiaries can also depend on the characteristics of the neighboring perennial plant (e.g. (Gómez-Aparicio et al. 2004) but can also change with extreme temporal fluctuations. Therefore, the net effect of facilitation may change along gradients of stress (Callaway and Walker 1997).

The inclusion of facilitation or positive species interactions into classical niche theory (Grinnell 1917; Hutchinson 1957) results in niche occupation by a species that exceeds that of its fundamental niche (i.e. the niche in the absence of biotic factors). Although at the macroscale, environmental gradients are commonly used to investigate trait differences between populations (Kurze et al. 2017), at the microscale the presence of perennial plants in a harsh landscape generates habitat heterogeneity and so creates buffered micro-refuges which can enhance the survival of species or ecotypes that are less adapted to the environment (Rull 2009; Soliveres et al. 2011). For example, the creation of different microhabitats underneath the canopies of perennial plants (Armas and Pugnaire 2005) can modify selection pressures at the microscale, resulting in trait differences at fine-grained spatial scales (Bergholz et al. 2017a) and has been shown to drive local adaptation to neighbors within populations (O'Brien and Schlüter 2020). Indeed, several studies have shown that the microhabitat underneath perennial plant cover can affect fitness related traits including higher levels

of fruit set, heavier seeds (Tirado and Pugnaire 2003) and differences in flowering (Halliday et al. 1994).

Interspecific trait variability represents the response of different species to a set of abiotic and biotic conditions, yet within-species trait differences can also have significant ecological impacts (Bergholz et al. 2017b; Bolnick et al. 2011). The Mediterranean *Brachypodium distachyon* complex, native to regions including Western Europe, the Middle East, Asia, and North Africa (Catalán and Olmstead, 2000; Schippmann, 1991), consists of three species *B. distachyon*, *B. stacei*, and *B. hybridum* the latter of which originated from the hybridization of *B. stacei* and *B. distachyon* (Catalán et al. 2012). Both *B. distachyon* and *B. hybridum* have been extensively studied in genetic experiments revealing diverse life history strategies influenced by traits such as flowering time, vernalization requirements, drought tolerance, and plant height (Schwartz et al., 2010; Woods et al., 2017; Manzaneda et al. 2015; Tyler et al., 2014). However, while studies have shown that neighboring plants can drive local adaptation among its beneficiaries (O'Brien and Schlüter 2020), it remains unclear whether neighboring perennial plants can drive ecotypic differences within *Brachypodium* populations. Whether or not influences from perennial neighboring plants may have the potential to shape functional diversity in annual grasses in semi-arid landscapes remains largely unexplored particularly in natural populations within the *Brachypodium distachyon* complex.

In this study, we focused on natural populations of *Brachypodium* and investigated 1) if they are differently associated with neighboring perennial plants as aridity increases, 2) if phenotypic traits were differently affected by perennial facilitation, and 3) if an association with neighboring perennial plants affected the phenology differentially in *Brachypodium* plants that were collected from the most arid and least arid sites.

Materials and methods

Study sites

We chose ten sites along an aridity gradient derived from the Global Aridity Index (GAI) in southeastern Spain (Table S1) (Zomer and Bossio 2007; Zomer et al. 2008): Cazorla (CZ), Tiscar (TI), Puente de la Sierra (PS), Hinojares (HJ), Fontanar (FO), Jodar (JO), Zujar (ZU), Fontanar Mirador (FM), Larva (LA), and Sorbas (SO). The sites are located in the Jaén and Almería provinces in southeastern Spain (Fig. 1) which include several less arid sites nested in the mountains and valleys of the Natural Park of Sierras de Cazorla, Segura y Las Villas, and surrounding smaller mountain ranges (Figs. S1, S2). The mean temperature for the 10 sites ranges from 12.9 to 16.6 °C, mean precipitation ranges from

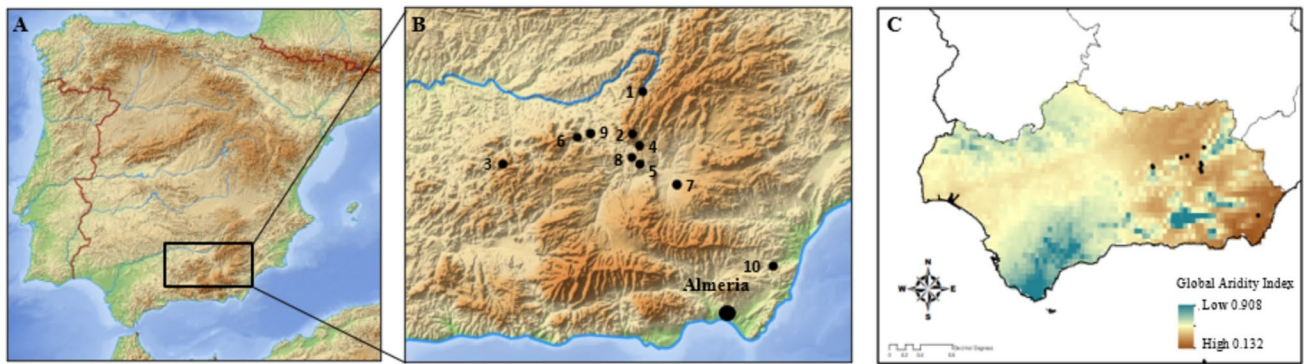


Fig. 1 **A** Region of Spain where the study was conducted. **B** Locations of plots in southeastern Spain. **C** Global Aridity Gradient derived for the 10 sites in the study

282 to 470 mm/year, mean maximum temperature ranges from 18.9 to 22.5 °C, mean minimum temperature ranges from 7.0 to 10.6 °C and site elevations range from 387 to 1192 m (Table S2). The prevailing direction, or aspect of the slope, as well as the steepness of the slope (slope percentage), were recorded per site (Table S2). The sites consisted of a patchwork of Mediterranean woody shrubs, perennial bunchgrasses, and sparsely vegetated open areas dominated by spring annuals and perennials (Armas et al. 2011), all showing some evidence of disturbance (cattle or goat trails in the site, steep slopes with erosion, or signs of past plowing). Populations or sites were dominated by either *Retama sphaerocarpa*, a leguminous shrub capable of hydraulic lift (Prieto et al. 2010), *Stipa tenacissima*, a native perennial bunchgrass known to compete intensively under low soil moisture conditions, or *Pinus pinaster*, a native tree that is commonly planted for soil stabilization in the mountains.

Aridity index

We assessed the aridity for the 10 sampling sites by utilizing the Global Aridity Index (GAI) of CGIAR-CSI (www.cgiar-csi.org; (Zomer and Bossio 2007; Zomer et al. 2008), a global database that contains mean monthly precipitation and potential evapotranspiration collected from 1950–2000 to a resolution of 1 km (30 arc per second). The GAI has a scale from 0 to 1 and an increase in GAI represents an increase in relative humidity and lower aridity (Zomer and Bossio 2007; Zomer et al. 2008). The GAI of the sites ranged from 0.168 (most arid site) to 0.290 (least arid site). We designed an aridity gradient to capture known physiological tolerance thresholds for *B. hybridum* and *B. distachyon* based on previous studies. This included a high-altitude site (> 1200 m) in the mountains, representing the least arid conditions (Catalan et al. 2016), and a low-altitude site with minimal precipitation, similar to populations in the most arid regions of southeastern Spain (Manzaneda

et al. 2012). Although the aridity gradient may seem rather short, such regional gradients have also been used in similar studies from southeastern Spain, e.g. Soliveres et al. (2011), and Maestre and Cortina (2004). Incremental changes in the Global Aridity Index can signal important ecological thresholds. For example, Berdugo et al. (2020) found that a 0.1 increase in aridity led to significant changes in plant diversity and productivity in semi-arid landscapes.

We also extracted the 50-year monthly means for the evapotranspiration index (ETO), GAI, mean precipitation, mean temperature, and mean minimum and maximum temperatures at the sites (Table S1).

Abiotic conditions

To investigate whether temperature and light intensity differed underneath in contrast to outside neighboring perennial plants, microclimate conditions were tested in a manageable subset of sites (SO, FM, HJ, FO, JO, LA, TI, CZ). However, owing to animal interference (probably mountain goats and wild boars), the loggers from TI and CZ were compromised and we were unable to use their data. Temperature and light intensity were recorded every hour with a pair of Hobo data loggers placed 5 cm above the soil underneath and outside of the canopy of one *R. sphaerocarpa* (the most dominant shrub at these sites) at each of the remaining six sites from May 9th to June 13th, 2018.

In May 2018, we took soil samples to measure soil moisture, but we were limited to only six of the ten sites (SO, TI, HJ, FO, JO, LA) as a rain event had occurred in the other four sites the day before and would have substantially affected measurements. Revisiting these 4 sites later was logistically not possible. From each of the six remaining sites, 10 total soil cores were taken. Five soil cores were taken from underneath five of the original 12 perennial plants which were paired with five cores taken outside perennial plant cover at each site. Each sample was placed in a

plastic bag in a cooler until returning to the lab and placed at 4 °C overnight. The next day, soil moisture was measured using the standard thermogravimetric technique (Black 1965) in which each soil sample was mixed and measured to a constant weight, placed in the oven at 60 °C for 48 h, and then re-weighed to calculate soil moisture content.

Spatial associations

We investigated whether *Brachypodium* was spatially associated with neighboring perennial plants at each site. Within each site, natural populations of *Brachypodium* co-occurred intermittently with perennial plants ranging from 0.5 to 5.0 m in height that provided a partially shaded understory. Other perennial plants that were common at the sites include *Genista spp.*, *Quercus ilex*, *Salvia rosmarinus*, *Thymus spp.* Between the dates of April 23 and June 16, 2018, we used stratified random sampling to select 12 perennial plant species, identified them to species, and recorded the height and the widest point of their canopy as a measure of perennial nurse plant size at each of the 10 sites. Under each perennial, we placed four 25 × 25 cm quadrats each in one of four directions (N, W, S, E) and paired them with four 25 × 25 cm quadrats placed 0.5 to 3 m outside of its canopy. We counted individual *Brachypodium* plants underneath and outside perennial plants within the four paired quadrats (total of 8 quadrats) for each of the 12 perennial plants. The counts in the four quadrats underneath and outside each of the 12 perennial plants were then combined for a site-level count (based on 48 quadrats underneath and outside perennials) and used to calculate the Relative Interaction Index (RII), a commonly used index to characterize the strength and direction of the spatial association between target plants (here *Brachypodium*) and neighboring perennial plants (Armas et al. 2004). The RII is calculated as $RII = [(count\ underneath\ perennial\ plants - count\ in\ open) / (count\ underneath\ perennial\ plants + count\ in\ open)]$. RII is a symmetrical linear metric bound by -1 which indicates a negative association and +1 which indicates a positive association, while 0 indicates neutral interactions.

Species discrimination

Because distinguishing between *B. hybridum* and *B. distachyon* is difficult in the field, we used a procedure to discriminate between these two species as outlined in the protocol from Contreras et al. (2017) (additional details can be found in Supplemental Information 1).

Phenotypic trait variation

To investigate whether phenotypic traits were different between *Brachypodium* collected underneath in contrast to

outside perennial plant canopy cover, mature fruiting plant samples were collected between May 26 and June 16, 2018, at 8 sampling sites (except FO and FM owing to incomplete samples as a result of damage during transport) (Table S3). Ten plants underneath and outside of the original twelve perennial plants per site were collected for a total of 240 plants per site. The plant material of each sub-population (underneath and outside) was placed in two separate large envelopes and stored at room temperature until measurement. We randomly selected 24 specimens from underneath and outside perennial plant cover from the field-collected specimens, in which between 9 and 22 plant samples from the 8 sites were useable (a complete sample and species identification from genotyping was possible). The other two sites (FO, FM) have genotypes for species proportion data but we do not have phenotypic trait data due to incomplete samples (Table S3). The phenotypic traits measured were chosen to evaluate fitness and included: the height of the tallest flowering stem, number of stems per plant, number of spikes per the main stem, number of spikes per plant, spike length (from the base of the first spikelet) without awns for the main stem, spike length without awns per plant, number of florets per spike, the total number of florets per plant, mean number of viable seeds and second floret lemma length. Seed viability was distinguished by the presence of a solid seed.

Flowering time

Because phenology is a life history trait that could indicate the presence of different ecotypes, we assessed flowering time in the F_1 generation in controlled conditions from the seeds collected in May and June 2018 from *B. hybridum* mother plants. We tested whether there were differences in flowering between the least arid and most arid site as well as within sites, and whether they originated either underneath or outside the canopy cover of a perennial plant. We chose only the most arid and least arid sites for flowering time differences. We randomly selected 20 samples from the initial collection made in May 2018 from each position (underneath vs outside perennial plant canopy) for a total of 40 mother plants from each of the two sites. Sample sizes were ultimately uneven for the following reasons. Distinguishing *B. hybridum* and *B. distachyon* in the field is nearly impossible, so species discrimination can also be confirmed by genotyping after sampling. Because *B. distachyon* turned out to be very rare at these two sites we could only test if there were differences in flowering underneath and outside in *B. hybridum*. Therefore, those samples were removed from this analysis thus reflecting the uneven sample sizes. To ensure simultaneous germination, we placed the seeds in Petri dishes on filter paper with distilled water and wrapped them with aluminum foil. The Petri dishes were placed in 8 °C

for 72 h and then placed at room temperature (20–22 °C) in the dark for 5 days. On the 6th day, the germinated seeds were acclimated to light for 2 to 3 days and were sown into pots. The soil medium consisted of perlite, sand, and soil (0.5:0.5:1). Plants were grown in a growth chamber at 20 °C, 50% relative humidity with 16 h light and 8 h dark. Plants were checked for flowering every day for 24 weeks.

Data analysis

We tested the effect of aridity (GAI) on the spatial association of *Brachypodium* (*B. hybridum* and *B. distachyon* combined, as they are difficult to identify to species level in the field) with perennial plants by using a linear mixed model from the lme4 package (Bates et al. 2014). In the full model, RII was the response variable, whereas site GAI (continuous) and the different perennial groups (PG, consisting of 4 levels; *Retama*, *Stipa*, *Pinus*, or PP, a general grouping for perennial plant species that were not either *Retama Stipa* or *Pinus*) and the interaction between GAI and PG were used as fixed effects. Since only one site (LA) had a southeastern aspect, and the rest were either north or south, we placed the site (LA) into the south-facing aspect group for this analysis. The random effect used was site identity. The reduction of marginal predictors in the full model utilized backward elimination for fixed and random effects using the step function within the lmerTest package in R (Kuznetsova 2017). All statistical analyses were performed using R version 3.6.0 (R Core Team 2020).

We tested whether the ten measured phenotypic traits, particularly the difference in traits between underneath and outside perennials, varied along the aridity gradient by using a linear mixed model with restricted maximum likelihood estimates from the lme4 package (Bates et al. 2014) separately for each trait. The response variable was one of the ten traits, and the fixed factors were GAI, position and the interaction between GAI and position, (underneath or outside perennial plant). Site identity was used as a random factor to control for the non-independence of plants measured from the same site. All traits were log10 transformed to meet the assumptions of homoscedasticity and normality.

We used linear models to test whether temperature increased, and soil moisture decreased along the aridity gradient. To test whether temperature, light intensity (lumens), and soil moisture differed underneath and outside of perennial plants, we used separate linear mixed-effects models that utilized either temperature, light intensity or soil moisture as the response variables. For temperature and light intensity, we tested only the fixed effect of position (underneath vs outside) at six sites and used logger ID as a random effect to control for the repeated measurements of the loggers. For soil moisture, we tested the fixed effects of GAI, and position, and added site as a random factor. The

reduction of marginal predictors in the full model utilized backward elimination for fixed effects using the step function within the lmerTest package in R (Kuznetsova 2017).

We investigated if there were differences in flowering time between the two most extreme sites (SO, CZ) and within these two sites. Because our data was censored (not all plants flowered), we fitted a Kaplan Meier survival curve using the KMSurv package (Klein 1981). The Kaplan Meier is a log-rank test that uses a non-parametric maximum likelihood estimator which considers the probability of an individual flowering beyond each time step, given that some plants did not flower during the experiment (censored data). In this model, the probability of flowering was the response variable and time (days) was the explanatory variable. We tested if there were differences in the probability of flowering between *B. hybridum* plants that had originated underneath vs outside perennial plants within both SO and CZ.

Results

Spatial association

We found that as aridity increases, the spatial association with perennial plants changed from mildly positive (RII=0.27) to negative (RII=−0.41) (Fig. 2), but this aridity effect on the spatial association was only slightly statistically significant (the final model which retained only GAI as the main predictor (apart from the random site effect, $p=0.033$) had $F_{1,8}=5.72$, $p=0.044$). The fixed effects of perennial group ($F_{3,27}=0.70$, $p=0.56$) and the interaction between GAI and perennial group ($F_{3,39}=0.44$, $p=0.73$) were not significant predictors in this model. Because the site and/or the perennials present in the site (which differed between sites) might have had a large influence on the relationship (Fig. 2), we removed sites one by one to test if the result of the model remained similar. In most cases, p -values became larger than 0.05 (Table S3), but remarkably, after removing FM, the result became much more strongly significant ($F_{1,103}=9.74$, $p=0.002$). The random site effect disappeared from the model, and the perennial group became very significant as well ($F_{3,103}=4.34$, $p=0.006$), but there was no interaction of perennial group with aridity ($F_{2,101}=0.27$, $p=0.76$).

Phenotypic trait variation

We found that plants collected from the field underneath perennials were taller, had more and longer spikes, and had a higher mean number of seeds and mean number of florets relative to plants from outside perennial canopy cover (Table 1). Because we suspected that differences in phenotypic traits were caused by the data of the most arid site

Fig. 2 Relationship between GAI (lower values indicate a higher aridity) and RII, the spatial association between *Brachypodium* and perennial plants (- indicating a negative association and + indicating a positive association). A decrease in GAI represents a decrease in relative humidity and higher aridity. The colors represent the dominant species occurring for each site, PG represents a perennial plant that was not one of the three dominant species (*Pinus*, *Retama*, *Stipa*)

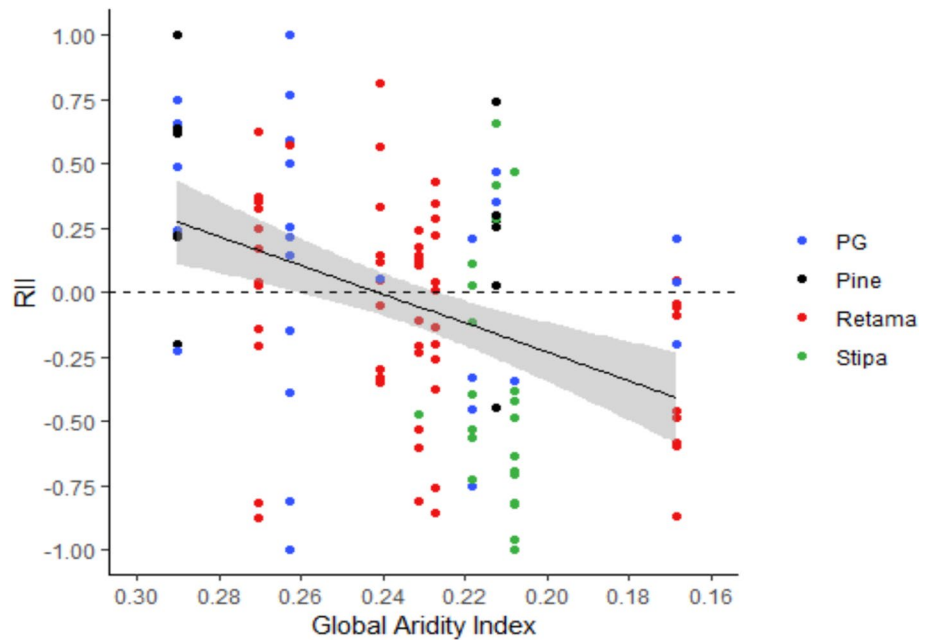


Table 1 Results of separate linear mixed models testing the fixed effects of global aridity (GAI), position (underneath vs outside) perennial canopy, and the interaction between GAI and position on each trait using Site as a random effect. Bold text indicates significance at $p < 0.05$

Traits	GAI			Position			GAI* Position		
	<i>F</i>	<i>d.f</i>	<i>P</i>	<i>F</i>	<i>d.f</i>	<i>P</i>	<i>F</i>	<i>d.f</i>	<i>P</i>
Stem height	3.35	1,6	0.12	62.7	1,203	< 0.001	1.18	1,202	0.28
Number of stems per plant	0.32	1,6	0.6	0.45	1,205	0.5	0.3	1,204	0.58
Number of spikes main stem	1.04	1,6	0.35	5.61	1,204	0.02	0.08	1,203	0.78
Number of spikes per plant	1.07	1,6	0.34	5.87	1,200	0.02	0.12	1,199	0.72
Spike length main stem	0.01	1,6	0.93	6.6	1,203	0.01	4.11	1,203	0.04
Spikelet length per plant	0.02	1,6	0.82	9.67	1,201	< 0.01	7.07	1,201	0.01
Number of florets main stem	1.2	1,6	0.32	5.15	1,201	0.02	1.18	1,200	0.28
Number of florets per plant	1.3	1,6	0.3	4.66	1,202	0.03	1.91	1,201	0.17
Number of viable seeds per plant	0.57	1,4	0.49	4.67	1,151	0.03	0.11	1,150	0.74
Mean palea length per plant	0.02	1,6	0.89	3.47	1,201	0.06	0.01	1,200	0.93

(SO), we repeated the analysis without this site; we still found that plants collected from underneath perennials were taller ($F_{1,162} = 39.0$, $p < 0.001$) and produced more spikes ($F_{1,163} = 6.28$, $p = 0.01$) but the higher number of seeds became statistically insignificant ($F_{1,110} = 3.78$, $p = 0.054$) and so did spike length per plant ($F_{1,161} = 2.88$, $p = 0.09$) (Table S4). Moreover, without the most arid site Sorbas (SO), the difference in phenotypic traits changed along the aridity gradient for spike length (main stem) ($F_{1,159} = 6.78$, $p = 0.01$) number of spikes per plant ($F_{1,163} = 6.28$, $p = 0.01$) and number of spikes per main stem ($F_{1,159} = 4.22$, $p = 0.04$) (Table S4). With the removal of Sorbas both the floret measures had significant interactions between location and GAI (number of florets per plant. $F_{1,160} = 7.5$, $p < 0.01$, and number of florets per main stem $F_{1,160} = 4.03$, $p = 0.046$. Mean spike length per plant no longer had a significant interaction (Table S4).

Flowering time

We found that *B. hybridum* at the most arid site (SO) flowered significantly earlier than *B. hybridum* at the least arid site (CZ) ($\chi^2(1) = 61.8$, $N = 112$, $p < 0.0001$). In addition, by 168 days, at the least arid site (CZ), we found within-population differences: the seeds that had originated outside of perennial plants yielded earlier flowering plants than seeds that had been collected underneath perennial plants. 30 out of 31 plants germinated from seeds that had originated outside of perennial plants flowered while 11 out of the 18 that had been collected underneath perennial plant cover flowered, a significant difference ($\chi^2(1) = 10.0$, $N = 49$, $p < 0.01$; see Fig. 3). We did not find significant differences between the probability of flowering at the most arid site (SO) at 12 weeks ($\chi^2(1) = 3.6$, $N = 63$, $p = 0.09$); by 14 weeks all plants (82/82) had flowered.

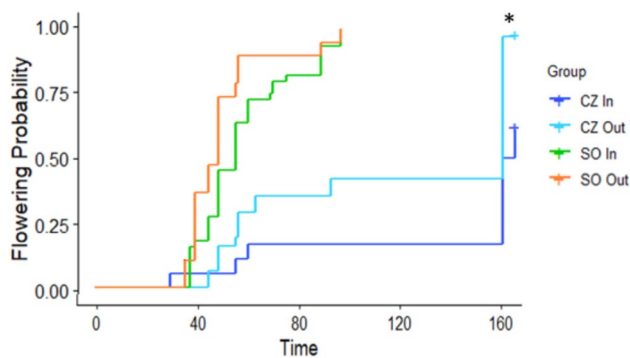


Fig. 3 Kaplan Meier survival curve representing the probability of flowering over time (days) from seed collected underneath (In) and outside (Out) perennial plant canopy cover collected from CZ=Cazorla (least arid) and SO=Sorbas (most arid). Asterisk (*) denotes $p < 0.05$ and indicates the significant differences between CZ In and CZ Out at 24 weeks and between *B. distachyon* and *B. hybridum* originating on the outside of perennial plant cover at SO

Abiotic conditions

We found that the mean site temperature did significantly increase as aridity increased ($t(4) = -4.05$, $p = 0.02$ but when we removed the arid site Sorbas (SO), temperature was no longer significant ($t(3) = -0.65$, $p = 0.56$). We also found that mean soil moisture did not significantly decrease with aridity ($t(4) = 2.44$, $p = 0.07$). However, at the plot level, we found that the abiotic conditions underneath the canopy cover of *R. sphaerocarpa* shrubs were different than those outside the canopy cover at the six sites measured. Temperature was significantly lower underneath than outside perennial plants for the six sites. ($t(10) = 3.86$, $p < 0.01$; $2.82 \pm 0.73 \text{ }^{\circ}\text{C}$) Fig. S3). Light intensity underneath perennial plant cover was significantly lower ($t(10) = 2.44$, $p < 0.05$; 2552 ± 1048) than outside canopy cover. Soil moisture was found to be significantly higher underneath perennial plants in contrast to the outside ($F_{1,53} = 5.86$, $p < 0.05$; 0.017 ± 0.001).

Discussion

The current study aimed to examine if neighboring perennial plants influence the occurrence of, and trait levels of, the related annual grasses *B. hybridum* and *B. distachyon* along an aridity gradient. We found that *Brachypodium* occurs less frequently underneath neighboring perennial plants (relative to outside such perennials) as aridity increases. Further, of all traits, only the difference in spike length between underneath and outside perennials changed along the aridity gradient. However, regardless of aridity, *Brachypodium* are taller, have more spikes, and have higher mean numbers of seeds and florets if they had originated underneath the canopy

of perennial plants in contrast to outside perennial plants. Finally, we found that flowering time was earlier from seeds collected from plants found outside the perennial plant canopy cover at the least arid site. Our results indicate that the annual *Brachypodium* is affected by local perennial plants and aridity plays a role in this, but the mechanisms behind the responses to the macroclimate (aridity) and the micro-habitat in semi-arid landscapes are multifaceted.

Spatial association

We found evidence for a negative relationship between the association of *Brachypodium* to perennial plants on the one hand and aridity on the other, with a significant contribution from the site as a random effect. Upon further analysis of this site effect by removing sites one by one, we found that one site, Fontanar Mirador, seemed to be mainly responsible because re-analysis without this site led to an insignificant site effect. Moreover, without this site included, the negative relationship between the association of *Brachypodium* to perennial plants and aridity was much more significant (much lower p -value of the null hypothesis), and also the perennial plant group became significant, but not their interaction. This means that the overall relationship between association and aridity is similar but with different offsets for different perennial plant groups.

Removing a site cannot be done lightly. However, when we chose the sites, FM was already the topic of discussion as it is very steep and has highly erodible soil. One would expect that the association with perennials would be more beneficial in such a site, and indeed as Fig. 2 shows, the RII is relatively higher in FM than one might expect from its aridity alone. The two other sites with similar aridity (Zujar and Larva) show a much smaller RII value, also for the same perennial plant (*Stipa*). Indeed on steep slopes, *S. tenacissima* tussocks can form small terraces of sediment upslope which consist of different nutrient levels and water filtration properties (Puigdefábregas 1996), and these ‘terraces’ have been found to increase facilitation of woody plants in south-eastern Spain (García-Fayos and Gasque 2002).

The effect of environmental gradients on the association of plant species with larger perennial neighbors in harsh landscapes is debated in the literature. Several studies find that negative interactions (Filazzola et al. 2020; Luzuriaga et al. 2012; Maestre and Cortina 2004) as well as positive interactions (Armas et al. 2011; Brooker et al. 2008) increase with stress, but others do not support this (Holmgren and Scheffer 2010; Maestre et al. 2005). Comparisons can be difficult between studies as diverse methods were used including different perennial plant species (Armas et al. 2011; Luzuriaga et al. 2012), beneficiaries (Armas and Pugnaire 2005; Holzapfel et al. 2006), and the span of the environmental gradient (Metz and Tielbörger

2016; Pugnaire et al. 2011). Co-existing species often respond differently to the same environmental conditions, experiencing varying levels of stress. In this study, the aridity gradient was a key factor expected to challenge *B. hybridum*, despite the potential stress, *B. hybridum* was continuously present, whereas the surrounding plant communities (dominated by *Retama*, *Stipa*, and *Pinus*) differed along the gradient. Additionally, the marked difference in flowering times that we found between the two most extreme sites likely captures early- and late-flowering ecotypes of *B. hybridum*. This observation aligns with Schwartz et al. (2010), who classified *Brachypodium* accessions into winter (late-flowering) and spring (early-flowering) types linked to genes influencing flowering time. This finding suggests that the aridity gradient in our study was strong enough to reveal adaptive responses in *B. hybridum* across the least and most arid sites. Our gradient is regionally located and therefore we use the mountainous landscape as a proxy to test lower levels of aridity in short distances. This necessarily introduces a confounding factor of elevation, but such confounding factors can rarely be avoided. Thus, as an annual species native to semi-arid landscapes, *B. hybridum* appears to exhibit local adaptation (flowering differences) with likely significant levels of phenotypic plasticity, enabling it to thrive under the stress gradient designed to challenge its resilience.

The response of the beneficiary to the perennial may depend on the characteristics of the perennial species (Gómez-Aparicio et al. 2004), slope aspect, and slope steepness, which changed along our aridity gradient, thereby potentially confounding our results. Smaller aridity gradients have also been used in the mountains of southeastern Spain which used variable slopes and soils (Maestre and Cortina 2004). Indeed, slope aspects can have different effects on soil moisture in semi-arid habitats (Gutiérrez-Jurado et al. 2013), but have been found to have limited effects on vegetation spatial aggregation (Alados et al. 2017), so we limited our study to equally distributed north and south facing slopes throughout the aridity gradient and allowed slope steepness to vary throughout our sites. As for the characteristics of specific perennial species, *R. sphaerocarpa* has been shown to alter soil properties and microbiota underneath its canopy possibly affecting the growth of annual plants (Lozano et al. 2017). *Pinus flexilis* has been found to facilitate the survival of planted shrubs and tree seedlings by decreasing wind speed (Baumeister and Callaway 2006). Further, *S. tenacissima* has been found to facilitate the growth of shrub seedlings underneath its canopy by increasing soil moisture and providing shade (Maestre et al. 2001). *Stipa* has a higher phylogenetic relatedness to *Brachypodium* and may be more likely to compete with it (Verdú et al. 2012), but on steep terrain with erodible soil the interaction might even be more positive for *Brachypodium* establishment.

The direction of biotic interactions can also change throughout the development of the beneficiary (Gasque and García-Fayos 2004; Schiffers and Tielbörger 2006). For example, proximity to a neighboring perennial plant can affect germination timing (Leverett 2017), increase germination success (Lozano et al. 2017), and affect seedling emergence and survival (Tielbörger and Kadmon 1995) and seedling densities (Holzapfel et al. 2006) which can affect the direction of the interactions. However, *B. hybridum* has been found to experience very high germination rates and seedling emergence regardless of the location of collection along a precipitation gradient, and the direction of the interaction may have been minimally affected early in development (Metz et al. 2018). Another likely scenario is competition between the perennial and beneficiary. For example, *S. tenacissima* has been found to facilitate *Pinus halepensis* as a juvenile but can turn into a competitor as the tree matures (Gasque and García-Fayos 2004). Other neighboring perennial plants can also indirectly affect the interactions between beneficiary species which can then affect the direction of the overall interaction (Aschehoug and Callaway 2015; Brooker et al. 2008). For example, Schob and colleagues found that although shrubs facilitated beneficiary species, the understory community was competing amongst itself, affecting traits such as plant mass and abundance (Schöb, 2013).

Phenotypic trait variation

Identification of mechanisms that combine spatial heterogeneity with trait variation could help guide the understanding of the role that biotic interactions play within habitats. Here, our question was whether neighboring perennial plants affect the traits of *Brachypodium* differentially along an aridity gradient. Although we did not find that aridity influenced trait values overall, we found differences in a majority of *Brachypodium* traits corresponding to the position (underneath or outside), and these differences changed along the aridity gradient for one trait (spike length). For the other traits, a majority was higher underneath than outside perennial plants, (e.g. including stem height, number of spikes, mean number of seeds per plant and mean number of florets per plant), likely pointing to the effects of neighboring perennial plants on the morphology of *Brachypodium*. This finding is also supported by the literature in which local microhabitat conditions can have a stronger effect on trait levels than environmental gradients (Bergholz et al. 2017a). Although there are no known studies comparing between and within-population phenotypic traits from field-collected samples in the *Brachypodium distachyon* complex, there are several studies investigating trait differences between populations from inbred germplasm lines (Catalán et al. 2012), F_1 generations in the greenhouse (Kurze et al. 2017), and

F₂ generations propagated in a common garden (Penner et al. 2019). In the short term, these differences in traits could be attributed to phenotypic plasticity. Although no evidence was found in this study, differences in morphological traits controlling reproduction (e.g. higher number of seeds) which we found in this study, could increase fitness, and over time, eventually drive local adaptation (ecotypic variation) within populations.

Flowering time

The flowering time of *B. hybridum* was significantly earlier in offspring from the most arid population in comparison to the least arid population. These results are in concordance with several studies that have conducted reciprocal transplants of *Brachypodium* showing that arid populations flower earlier than the Mediterranean populations (Liancourt and Tielbörger 2009; Penner et al. 2019).

In the least arid site, we found that plants from seeds that had been collected outside perennial plants flowered more quickly by week 24 than those from underneath perennials. A significantly different microhabitat that occurs underneath perennial plants (e.g. decreased light intensity, higher levels of soil moisture and fertility Lozano et al. 2017; Montesinos-Navarro et al. 2016)) in this landscape could lead to intraspecific differences in flowering. Another possibility is that several different ecotypes may be present within populations—whether from long-distance seed dispersal (Vogel et al. 2009) or from local adaptation (Michalet et al. 2011) and may have different flowering times. Although conducted in controlled conditions, this phenology study represents the only study that we are aware of that has investigated the possibility of neighboring perennial plants affecting flowering in *Brachypodium* occurring either underneath or outside canopy cover within one site. The next step would be to investigate if this is indeed present in natural populations.

This study is a snapshot in time, but overall, our findings support that in patchy landscapes dominated by perennial plants, the Mediterranean *Brachypodium distachyon* complex responds phenotypically and somewhat phenologically, to the presence of neighboring perennial plants. While the association with perennials decreases significantly with aridity, the role of aridity on this response is limited. We suggest that both the effects of aridity on association and the neighborhood effects on phenotypic traits of annual grasses should be more frequently incorporated into the evaluation of functional diversity within plant communities. Future research should investigate the evolutionary implications of positive interactions and explore whether the differences that we found in traits and along the aridity gradient, translate into differing levels of genetic variation that may lead to ecotypic variation within populations.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11258-025-01508-y>.

Acknowledgments This work was supported by the University of Groningen Adaptive Life Program (GELIFES). We would like to thank Yvonne Verkuil and Marco van der Velde for help with the genotyping, Laura Roldán Lloret and James Skinkis for assisting with plant measurements, and Teresa Salido for technical assistance in the lab. We would also like to thank Johannes Metz for your insightful and constructive feedback which greatly improved this manuscript as well as the anonymous reviewers for their valuable comments and suggestions.

Author Contribution MKK, CS, RSE, LVD: Conceptualization; MKK, AJM, LMM: Methodology; MKK, CS: Formal analysis; MKK, TAP: Investigation; MKK: Writing the original draft. All authors contributed to reviewing and editing the final draft.

Data Availability Data provided in support of this manuscript has been deposited in the Dataverse.NL public repository: <https://dataverse.nl/dataverse/GELIFES>.

Declarations

Conflict of interests The authors declare no competing interests.

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