UV radiation increases flavonoid protection but decreased reproduction in *Silene littorea*

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Abstract

Plants respond to changes in ultraviolet (UV) radiation via morphological and physiological changes. Among the variety of plant UV-responses, the synthesis of UV-absorbing flavonoids constitutes an effective non-enzymatic mechanism to mitigate photoinhibitory and photooxidative damage caused by UV stress, either reducing the penetration of incident UV radiation or acting as quenchers of reactive oxygen species (ROS). In this study, we designed a UV-exclusion experiment to investigate the effects of UV radiation in *Silene littorea*. We spectrophotometrically quantified concentrations of both anthocyanins and non-anthocyanin flavonoids (flavones) in petals, calyces, leaves and stems. Furthermore, we analyzed the UV effect on the photosynthetic activity in hours of maximum solar radiation and we tested the impact of UV radiation on male and female reproductive performance. We found that anthocyanin concentrations showed a significant decrease of about 20% with UV-exclusion in petals and stems, and 30% in calyces. Flavone concentrations showed a significant reduction of approximately 25% in calyces and stems, and 12% in leaves. Photochemical efficiency of plants grown under UV stress decreased sharply at maximum light stress, but their ability for recovery after light-stress was not affected. In addition, exposure to UV radiation does not seem to affect ovule production or seed set, but decreases the total seed production per plant and pollen production by 69% and 31%, respectively. Our results demonstrate that UV radiation produced opposite effects on flavonoid accumulation and reproduction in *S. littorea*. UV stress increased flavonoid concentrations, suggesting a photoprotective role of flavonoids against UV radiation, but

had negative consequences for reproduction. We propose that this trade-off helps this species to occupy exposed habitats with high UV radiation.

Keywords: abiotic stress, anthocyanins, Caryophyllaceae, flavones, male and female reproductive performance, photoprotection, photosynthesis, secondary metabolites, UV radiation.

Introduction

Ultraviolet (UV) radiation can both help and harm plants. Many flowering plants rely on UV nectar guides for pollination services [1]. Simultaneously, the high energy of UV radiation can be damaging to cells and presents a unique abiotic challenge to most land plants [2]. Furthermore, the "invisible" nature of UV radiation makes it particularly enigmatic at the ecological and physiological scales. UV-A (315 – 400 nm) and UV-B (280 – 315 nm) radiation has numerous positive and negative effects at the cellular and organismal scales [3–6], inducing a variety of morphological responses in plants [4,7]. In addition, UV-B radiation exerts damaging effects on DNA and chloroplasts, particularly photosystem II (PSII), and indirectly generate reactive oxygen species (ROS) that can further damage the photosynthetic apparatus [8,9].

Plants have developed a variety of mechanisms to avoid the harmful effects of UV radiation, mainly through the screening of UV wavelengths, the repairing of UV-induced damage or the quenching of ROS [6,8,9]. Although the latter is primarily performed by antioxidant enzymes that control ROS levels [8,10], flavonoids and other phenolic compounds are involved in the detoxification of ROS as well [11–13]. Flavonoids are potent scavengers of ROS that prevent lipid peroxidation and scavenge free radicals, especially those flavonoids having a catechol group in the B-ring of the flavonoid skeleton (e.g. quercetin derivatives) [14,15]. Furthermore, exposure to excess light or UV-B radiation increases the synthesis of effective antioxidant dihydroxy B-ring-substituted flavonoids (e.g. luteolin derivatives) at expense of other less effective antioxidant flavonoids (e.g. kaempferol derivatives) [16,17]. In addition to their key antioxidant functions, other studies

have attributed to flavonoids an important role in photoprotection through the screening of the UV radiation [18,19]. For example, epidermal flavonols play a predominant role in UV-B screening in leaves of *Secale cereale* and *Centella asiatica* [20,21].

Within the flavonoids, anthocyanins are plant pigments that are synthesized in the last steps of the flavonoid biosynthetic pathway [22]. Anthocyanins mainly absorb in the green region of visible (VIS) spectrum (500-565 nm), reducing the overall photosynthetically active radiation (PAR) (400 - 700nm) hitting the chloroplasts and helping plants to have a faster photosynthetic recovery after saturating light stress [23–25]. In addition, when anthocyanins are acylated, they can absorb UV radiation, and may contribute to ROS scavenging even more than other phenolic compounds [26–29]. Yet, UV stress is known to induce anthocyanin biosynthesis, which may contribute to the tolerance to UV radiation [30,31].

The aforementioned photoprotective functions of UV-induced flavonoids are not restricted to photosynthetic tissues, but also occur in floral structures such as anthers, ovaries, petals and sepals. Pollen grains accumulate flavonoids to protect them from UV-B damage and preserve their viability after anthesis [32], whereas flavonoids protect ovules by shielding ovaries from UV radiation [33]. In the same way, the accumulation of protective flavonoids in petals and sepals can reduce the damaging effects of UV radiation on these and other nearby reproductive tissues [34]. Additionally, petal flavonoids form a UV pigmentation floral pattern to guide pollinators to nectaries, thus UV-induced changes in the size of nectar guides might affect the pollination activity [1,35]. In addition, UV radiation may induce a variety of plant morphological responses in these reproductive structures. Many studies have reported a complex UV dose-response involving differential effects on reproductive morphological traits (reviewed in [7]). For example, Koti et al. reported that

86 UV-B radiation negatively affected the flower phenology, pollen germination and its viability
87 in soybean (*Glycine max*) [36], and similarly decreased pollen and flower production over
88 time in *Brassica rapa* [37].

89 This paper describes the effects of UV-radiation in flavonoid accumulation and
90 reproduction of the shore campion (*Silene littorea* Brot., Caryophyllaceae). This annual
91 species is endemic to coastal foredunes along the Iberian Peninsula and accumulates
92 flavonoids (flavones and anthocyanins) in petals, calyces, stems and leaves [38,39]. Our
93 previous work has shown a latitudinal gradient in flavonoid accumulation that tends to
94 increase from north to south in most plant tissues - correlated with increased solar exposure
95 and temperatures [39]. Moreover, we found that intense solar radiation, including UV and
96 VIS spectra, increased the synthesis of flavones and anthocyanins in most aboveground
97 tissues of *S. littorea* [40]. In this study, we focus on the effect of the UV irradiation on
98 flavonoid accumulation in this species. We quantified the concentrations of flavones and
99 anthocyanins in petals, calyces, leaves and stems of plants grown with or without exposure
100 to UV radiation. Then, we analyzed the UV effects on the photosynthetic efficiency and the
101 male and female reproductive output.

102 Flavonoids have a key role in photoprotection [15,19], but the synthesis of these
103 compounds may represent a cost for the plant [24]. Consequently, we predict that the
104 exclusion of UV radiation will result in a decrease in UV-inducible flavonoid concentrations in
105 all tissues. This energetic and carbon savings under UV-exclusion may result in increased
106 reproductive allocation [41]. In contrast, without UV protection, we predict that
107 photodamage will decrease photosynthetic activity [9,42] and show lower reproductive
108 output. Since *S. littorea* inhabits exposed coastal dunes habitats with high solar radiation

levels, we hypothesize that this species will have effective light-stress recovery system that prevents long-term photoinhibition.

Materials and methods

Study system and experimental design

Silene littorea is an annual plant that accumulates anthocyanins (cyanidin derivatives) and flavones (mainly isovitexin and isoorientin derivatives) in both reproductive and vegetative tissues [38] (Fig 1). This species inhabits coastal populations from the northwestern corner to the southeastern portion of the Iberian Peninsula [39]. We collected seeds of six plants from a northwestern population (Furnas; 42° 38' 15'' N, 9° 2' 21'' W) and six plants from a southwestern population (Sines; 37° 55' 17'' N, 8° 48' 17'' W). These two populations, which are separated by approximately 500 km along the Atlantic coast of the Iberian Peninsula, are exposed to a different degree of solar irradiance, being approximately 30% higher in southern latitudes [39].

Fig 1. Details of a *Silene littorea* plant (A) to show the accumulation of anthocyanins throughout the whole plant. B, C and D showed stereo-microscope photographs of surface of the calyx ribs, adaxial surface of the leaf, and cross section of a stem, respectively. Scale bar: 5 mm (A), 0.5 mm (B, C), and 1 mm (D).

Seeds obtained from the 12 maternal families were scarified and maintained at 45 °C for a month to break dormancy, and afterwards they were germinated in a germination

chamber at 22 °C/15 °C (12 h light/12 h dark). The resulting seedlings were planted in pots filled with 2.5 L of a mixture of standard substrate (80-90% organic material, pH = 6.5) and beach sand (v:v 50:50) and were grown in the greenhouse at Pablo de Olavide University (Seville, Spain) for one month. In February 2016 (one month before flowering), pots were put outside on two benches in the experimental garden. The bench assigned to the UV-present treatment was covered with a methacrylate filter that transmitted 100% UV irradiance, whereas the bench assigned to the UV-exclusion treatment was covered with a polycarbonate filter preventing most UV radiation (< ~ 385nm). The UV-exclusion treatment produced a reduction of 9.2% and 100% of total transmitted sunlight and UV radiation, respectively. Maximum solar irradiation at natural sunlight was 1258 W/m^2 and UV irradiance was 4.36 W/m^2. Measures were taken at 14:00 h of a sunny day (6^{th} June 2016). Total solar radiance and UV were measured by means of Megger PVM210 irradiance meter (range sensitivity = 1999 W/m^2; resolution = 0.1 W/m^2) (Megger Co., Dallas, USA) and PCE-UV34 UV light meter (range sensitivity = 0.000 to 19.9 W/m^2; resolution = 0.01 W/m^2) (PCE Inst., Durham, UK), respectively. Given the low germination rates of this species and the high mortality at the seedling stage, the final number of surviving plants was 65 (belonging to nine maternal families). These plants were assigned to the UV-present (41) and UV-exclusion (24) treatments, respectively (S1 Table). Plants that shared the same maternal family were equally assigned to each treatment whenever possible.

Flavonoid quantification

During peak flowering (May 2016), samples of petals, calyces (five petals and the calyx of the same flower), leaves (selected mid-stem) and stems (1 cm length section from

the main axis) were collected from 34 and 22 plants grown in the UV-present and UV-exclusion environments, respectively (S1 Table). Samples were extracted in 1.5 ml of MeOH containing 1% of HCl following the procedure described in Del Valle et al. [39]. Three replicates of 200 µL per sample extraction were used to estimate flavonoid concentrations using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Anthocyanins and flavones were quantified at A_{520} and A_{350}, respectively. In photosynthetic organs (calyces, leaves and stems), anthocyanin concentration was corrected as A_{520} - (0.24 x A_{653}) to compensate for the small overlap absorption by chlorophyll [43]. Anthocyanins and flavones concentrations were calculated following Del Valle et al. [38] and expressed as milligrams of cyanidin-3-glucoside, isovitexin and isoorientin equivalents per gram fresh weight, respectively.

Assessment of photosynthetic activity

To determine if there were physiological consequences of plants grown with and without UV radiation, the photochemical efficiency of PSII (*Fv/Fm*) was measured in calyces and leaves of 30 plants from Sines (14 and 16 from the UV-present and UV-exclusion treatments, respectively) using a field portable pulse-modulated chlorophyll fluorometer (FMS2, Hansatech Instruments, Norfolk, UK). Measurements were carried out in predawn (~ 0700h) and in maximum solar radiation (~1430h), and in the early (March) and maximum (May) stages of the flowering period. To asses the physiological status of photosynthetic tissues across the experiment, measurements were carried out in fully exposed plants on two sunny days [44]. To minimize temporal variation in *Fv/Fm*, all measurements were made

in a period of one hour. Prior to taking physiological measurements, samples were acclimated for 30 minutes in dark using leaf-clips that contain a mobile shutter.

Assessment of plant reproductive performance

Flower and fruit production in 41 and 24 plants from the UV-present and UV-exclusion treatments were monitored weekly during the entire flowering period, from March 10th to June 20th. These individual flowers were monitored for either fruit production or fruit abortion to determine the proportion of flowers that set fruit. In May, one mature fruit per plant was collected if possible. A total of 33 and 21 mature fruits were collected from plants growing in the UV-present and UV-exclusion treatments, respectively. For each mature fruit, their seeds and aborted ovules were counted under the dissecting microscope to calculate the proportion of ovules that set seed. Then, seed production per plant was estimated for those plants from which we collected mature fruits and calculated as the product of seeds/fruit x total number of fruits produced during the flowering period. Pollen and ovule production were analyzed following the procedure described in Narbona et al. [45] from unopened flower buds preserved in FAA (95 % ethanol, dH_2O, 37-40 % formaldehyde, acetic glacial acid, 10:7:2:1) of nine and 13 plants grown in the UV-present and UV-exclusion treatments, respectively. The total number of pollen grains per anther was calculated as the average of pollen grains counted in one upper and one lower anther of an unopened flower bud per plant.

Statistical analysis

Generalized linear mixed models (GLMMs) with Gaussian link functions were used to test the effect of UV radiation on the accumulation of anthocyanins and flavones in each plant tissue, considering treatment and population as fixed factors and maternal family as a random factor. Flavonoid concentrations were log-transformed prior to conduct the GLMMs analysis. Pairwise comparisons between UV-present and UV-exclusion treatments were carried out using the "multcomp" R-package and its "cld" (compact letter display) function was used to show differences between populations [46]. Due to the low number of experimental plants, we used the conservative Bonferroni adjustment of p-values in pairwise comparisons [47]. The same analyses were used to test for differences in male and female reproductive performance and in the photochemical efficiency of PSII (*Fv/Fm*) between plants grown in the different UV treatments. For this latter analysis, independent comparisons were done for leaves and calyces and in the early (March) and maximum (May) stages of the experiment, as well as pairwise comparisons of the photochemical efficiency between predawn and afternoon conditions. Pearson's correlations with a Bonferroni adjustment for multiple comparisons were used to assess the relationship between flavonoid production and male and female reproductive output [48]. All analyses were performed in R v3.4.0 [49]. GLMMs were carried out using the R library "lme4" [50].

Results

Effects of UV radiation on flavonoid production

In general, plants from the UV-exclusion treatment showed lower accumulation of anthocyanins, but this decrease was not homogenous in all tissues. Specifically, anthocyanin

220 concentrations in petals and stems decreased approximately 20% in these plants, whereas in
221 calyces the decrease was of 30% and the differences were marginally significant (Fig 2, Table
222 1). Anthocyanins were nearly absent altogether in leaves (Fig 2E). Flavone concentrations in
223 plants from the UV-exclusion treatment were lower by 12%, 23%, and 25% in leaves, calyces,
224 and stems, respectively, but in petals the differences were not significant (Table 1).
225
226 **Fig 2. Boxplots representing anthocyanin and flavone concentrations in the UV-present**
227 **(purple boxes) and UV-exclusion (white boxes) treatments in petals (A, B), calyces (C, D),**
228 **leaves (E, F) and stems (G, H).** The central line displays the median, the bottom and top of
229 the box are the first and third quartiles, and dots represent sample values. Lowercase letters
230 are used to show results of multiple comparisons between populations. Within each
231 population, pairwise comparisons between light treatments using Bonferroni adjustment are
232 showed. FW, fresh weight; ns, not significant; ns*, marginally significant; *, $P < 0.05$; **, $P <$
233 0.01; ***, $P < 0.001$.
234

Table 1. Results from GLMMs testing the effect of UV radiation, population and their interaction on the production of anthocyanins and flavones in each plant tissue.

		Anthocyanins					Flavones				
Tissue	**Source of variation**	**SS**	**Numerator d.f.**	**Denominator d.f.**	***F***	***P***	**SS**	**Numerator d.f.**	**Denominator d.f.**	***F***	***P***
Petals	Treatment	0.670	1	46.62	7.968	**0.007**	0.019	1	38.78	1.469	0.233
	Population	0.033	1	19.79	0.396	0.536	0.477	1	42.86	36.15	**<0.001**
	Treatm. x Pop.	0.096	1	47.86	1.140	0.291	0.001	1	38.68	0.004	0.949
Calyces	Treatment	0.674	1	44.48	3.729	0.059	1.013	1	44.39	37.27	**<0.001**
	Population	0.121	1	43.22	0.667	0.419	0.606	1	46.06	22.29	**<0.001**
	Treatm. x Pop.	0.231	1	45.11	1.276	0.265	0.018	1	44.94	0.647	0.426
Leaves	Treatment	0.007	1	46.70	0.176	0.677	0.368	1	49.00	5.145	**0.028**
	Population	0.078	1	23.18	1.899	0.181	1.166	1	49.00	16.28	**<0.001**
	Treatm. x Pop.	0.199	1	48.29	4.840	**0.033**	0.023	1	49.00	0.327	0.570
Stems	Treatment	1.572	1	44.65	8.527	**0.005**	1.295	1	46.10	6.203	**0.016**
	Population	0.035	1	49.27	0.191	0.664	0.049	1	42.77	0.237	0.629
	Treatm. x Pop.	0.339	1	45.22	1.837	0.182	0.008	1	46.92	0.041	0.841

Significant *P*-values were highlighted in bold.

235

Sines and Furnas populations did not show significant differences in anthocyanin concentrations in any of the sampled tissues (Table 1). Conversely, the flavone concentrations were significantly higher in plants from Sines, the southern population, in all tissues except for the stems (Fig 2, Table 1), and the interactions of light treatment and population were not significant (i.e. the decrease of flavone concentration in UV-exclusion plants was homogeneous in both populations).

When we analyzed each population independently, we found that the only significant differences in anthocyanin concentrations between treatments were in petals of plants from Furnas and in stems of plants from Sines (Figs 2A and G). With respect to flavones concentrations, the only significant differences between treatments were found in calyces of plants from both populations (Fig 2D). Interestingly, plants from both UV-exclusion and UV-present treatments of Sines showed higher levels of flavones than their respective treatments in Furnas.

Effects of UV radiation in photosynthetic performance

Plants decreased their photochemical efficiency (*Fv/Fm*) in the afternoon, when plants were maximally exposed to light stress, but in the predawn, after a whole night of relaxation of photoinactivation, they showed *Fv/Fm* values within the range of healthy plants (~ 0.85) (Fig 3, Table 2). Leaves showed significant differences in their photochemical efficiency between UV-treatments and between measurement conditions (predawn or afternoon), and the interaction of UV-treatments and measurement conditions was also significant (Table 2). In the afternoon, leaves of the UV-exclusion treatment showed a 20.8% and 57.4% reduction of *Fv/Fm* values in early (March) and maximum (May) stages of the

flowering period, respectively ($P < 0.001$ for both pairwise comparisons, Table 3; Fig 3A and B). In calyces, statistical differences in their photochemical efficiency were found only between measurement conditions (predawn or afternoon) both in March and May (Table 2). Pairwise comparisons in calyces revealed significant lower *Fv/Fm* values in afternoon conditions, regardless of the UV treatment or the flowering period ($P < 0.032$, Table 3; Fig 3C and D).

Fig 3. Variation of photochemical efficiency (*Fv/Fm*) from predawn conditions to afternoon. The mean *Fv/Fm* values obtained from leaves (A, B) and calyces (C, D) in the early (March; A and C) and maximum (May; B and D) stages of the flowering period are showed. Plants from the UV-present treatment are displayed by pink filled circles and solid lines, whereas those from the UV-exclusion treatment are displayed with empty circles and dashed lines. Results of independent pairwise comparisons after Bonferroni corrections between UV treatments in predawn and afternoon conditions are displayed. Error bars represent ± SE.

Table 2. Results from GLMMs testing the effect of UV radiation, measurement condition (predawn or afternoon) and their interaction on the photochemical efficiency of PSII (*Fv/Fm*) in leaves and calyces.

Tissue	Stage	Source of variation	SS	Numerator d.f.	Denominator d.f.	*F*	*P*
Leaves	Early (March)	Treatment	0.030	1	50.00	19.07	**< 0.001**
		Measurement condition	0.056	1	50.00	34.86	**< 0.001**
		Treatm. x Measurement condition	0.020	1	50.00	12.34	**< 0.001**
	Maximum (May)	Treatment	0.083	1	40.00	6.209	**0.017**
		Measurement condition	0.528	1	40.00	39.50	**< 0.001**
		Treatm. x Measurement condition	0.115	1	40.00	8.614	**0.006**

Calyces	Early (March)	Treatment	0.004	1	32.39	2.822	0.103
		Measurement condition	0.039	1	47.25	30.42	**< 0.001**
		Treatm. x Measurement condition	0.004	1	47.25	3.401	0.071
	Maximum (May)	Treatment	0.001	1	40.39	0.182	0.672
		Measurement condition	0.375	1	38.80	48.32	**< 0.001**
		Treatm. x Measurement condition	0.005	1	38.80	0.639	0.429
Significant *P*-values were highlighted in bold.							

275

Table 3. **Comparisons of the photochemical efficiency (*Fv/Fm*) between predawn and afternoon conditions.** Pairwise comparisons were independently performed in leaves and calyces from the UV-exclusion and UV-present treatments and either in the early (March) and maximum (May) stages of flowering period.

Tissue	Stage	Treatment	Estimate	Std. Error	*Z* value	*P*
Leaves	Early (March)	UV-exclusion	-0.027	0.014	-1.873	0.239
		UV-present	-0.104	0.017	-6.117	**< 0.001**
	Maximum (May)	UV-exclusion	-0.117	0.047	-2.480	0.063
		UV-present	-0.323	0.052	-6.252	**< 0.001**
Calyces	Early (March)	UV-exclusion	-0.037	0.013	-2.788	**0.032**
		UV-present	-0.073	0.015	-4.889	**< 0.001**
	Maximum (May)	UV-exclusion	-0.163	0.035	-4.611	**< 0.001**
		UV-present	-0.205	0.039	-5.202	**< 0.001**
Significant *P*-values were highlighted in bold.						

276

277 ## Effects of UV radiation in reproductive performance

278 Flower production showed statistical differences between the two experimental
279 conditions (Table 4). Plants from the UV-exclusion treatment displayed approximately five
280 times more flowers than those with UV-present (261.4 ± 30.1 and 50.7 ± 8.3, respectively;
281 mean ± SE; Fig 4A). In addition, flower production was significantly different for both
282 populations, being higher in Sines plants. Conversely, fruit set was statistically higher in the
283 UV-present treatment and in plants from Furnas population (Fig 4B, Table 4). The number of

284 ovules per flower and seed set was statistically similar between light treatments or
285 populations (Figs 4C and D). The total seed production per plant was approximately three
286 times higher in plants from the UV-exclusion treatment compared to the UV-present plants
287 (46.2 ± 7.8 and 14.3 ± 1.9, respectively; Fig 4E), and did not show statistical differences
288 between populations (Table 4). Pollen production decreased by ~31% in plants exposed to
289 UV radiation (2126.1 ± 99.0 and 1473.9 ± 85.8, respectively; Fig 4F), but again differences
290 between populations was not found. The interactions of UV treatment and population were
291 not significant for any of the studied reproductive outputs (Table 4).
292
293 **Fig 4. Boxplots representing the total flowers per plant (A), fruit set (B), ovules per flower**
294 **(C), seed set (D), seed production per plant (E) and pollen per anther (F) in plants growing**
295 **in the UV-present (purple boxes) and UV-exclusion (white boxes) treatments.** Dots
296 represent values for all estimations of plant reproductive performance. The central line
297 displays the median, the bottom and top of the box are the first and third quartiles, and dots
298 represent sample values. Letters displays are used to show results of multiple comparisons
299 between populations. Within each population, pairwise comparisons between light
300 treatments using Bonferroni adjustment are showed. ns, not significant; *, $P < 0.05$; **, $P <$
301 0.01; ***, $P < 0.001$.
302

Table 4. Results from GLMMs testing the effect of UV radiation, population and their interaction on the estimations of male and female reproductive performance in *S. littorea*.

	Source of variation	SS	Numerator d.f.	Denominator d.f.	*F*	*P*
Flowers per plant	Treatment	37.55	1	52.45	58.04	**< 0.001**
	Population	5.040	1	6.604	7.789	**0.029**
	Treatm. x Pop.	0.648	1	60.01	1.002	0.321

Fruit set	Treatment	1.457	1	61.00	9.476	**0.003**
	Population	2.405	1	61.00	15.64	**< 0.001**
	Treatm. x Pop.	0.112	1	61.00	0.725	0.398
Ovules per flower	Treatment	0.020	1	12.21	0.524	0.483
	Population	0.050	1	17.02	1.282	0.273
	Treatm. x Pop.	0.007	1	12.55	0.183	0.677
Seed set	Treatment	0.056	1	48.29	0.267	0.608
	Population	0.001	1	27.29	0.009	0.924
	Treatm. x Pop.	0.140	1	49.75	0.666	0.418
Seeds production per plant	Treatment	11.42	1	50.00	21.48	**< 0.001**
	Population	0.401	1	50.00	0.755	0.389
	Treatm. x Pop.	0.012	1	50.00	0.022	0.883
Pollen per anther	Treatment	0.616	1	17.00	25.32	**< 0.001**
	Population	0.004	1	17.00	0.158	0.696
	Treatm. x Pop.	0.093	1	17.00	3.807	0.067

Significant *P*-values were highlighted in bold.

When assessing the relationship between flavonoid production and male and female reproductive outputs, we did not found any significant correlations at Bonferroni-corrected level ($\alpha = 0.05/6 = 0.008$) (S2 Table).

Discussion

Effects of UV radiation on flavonoid production

Exposure to UV radiation led to a generalized increase in the anthocyanin and flavone concentrations in *S. littorea,* suggesting that increasing flavonoid concentrations is part of this plant's response to UV stress. In this species, all anthocyanins in aboveground tissues are cyanidin derivatives, whereas the most abundant flavones are apigenin derivatives (isovitexin) in petals and luteolin derivatives (isoorientin) in photosynthetic tissues [38].

315 Isoorientins are dihydroxy B-ring-substituted flavonoids, which it is known to have higher
316 antioxidant properties [14,51–53]. In this regard, the UV-induced accumulation of efficient
317 antioxidant flavonoids has been previously described in plants. For example, the flavonoid
318 composition in leaves of the white clover (*Trifolium repens*) is affected by solar radiation, as
319 the concentration of quercetin increases under UV-B stress rather than those of less
320 effective antioxidants such as monohydroxylated kaempferol glycosides [54]. In addition,
321 most anthocyanins and flavones in *S. littorea* are acylated [38], which it is known to enhance
322 the flavonoid absorption in the UV-A and UV-B wavelength [28,55,56]. In *Cistus salvifolius*,
323 for example, the occurrence of acylated flavonoids in trichomes ameliorates stress from
324 excess UV radiation [57], whereas leaves of purple basil (*Ocimum basilicum*) accumulate
325 coumaroyl anthocyanins that are more responsive to quenching sunlight irradiance, mainly
326 in the UV-B wavelength, than non-acylated anthocyanins [27]. Although we did not obtain
327 direct evidences of variations of the flavonoid-mediate quenching of UV-induced free
328 radicals in *S. littorea*, its flavonoid profile, including acylated and efficient antioxidant
329 flavonoids, suggests that this species show a robust biochemical toolkit that may protect
330 itself from the oxidative stress caused by UV radiation.

331 Despite differences in flavonoid production caused by UV radiation, plants not
332 exposed to UV light accumulated important amounts of anthocyanins and flavones in most
333 aboveground tissues. This may be due to the incidence of high levels of PAR on these plants.
334 Several studies indicate that UV irradiance is not a prerequisite for flavonoid biosynthesis.
335 For example, the concentration of UV-absorbing quercetin in *Brassica oleracea* increases in
336 line with PAR levels [58]. In *Arabidopsis thaliana*, PAR-only exposure contributes to the
337 formation of a base amount of quercetin, providing a basic photoprotection that is further

338 increased by long term exposure to UV-A/B radiation [59]. Similarly, high levels of PAR might
339 lead to the formation of a base amount of anthocyanins and flavones in *S. littorea*, whose
340 concentrations could be optimized and increased when plants are exposed to UV radiation.
341 However, given that anthocyanins and flavones perform a plethora of protective functions
342 against many biotic and abiotic factors [28,60,61], they could be performing non-
343 photoprotective functions. For example, isoorientins and isovitexins found in flax (*Linum*
344 *usitatissimum*) enhance the resistance to fungal infections [62], and petal isovitexins of
345 *Silene latifolia* help regulate vacuole homeostasis in epidermal cells, preventing petals from
346 wilting [63]. In addition, flavones are produced constitutively in aboveground tissues of *S.*
347 *littorea* when plants grow in low light levels conditions [40]. Thus, although anthocyanins
348 and flavones are probably conferring protection against high levels of PAR, we cannot rule
349 out that the selective pressures of other biotic and abiotic agents could explain the
350 constitutive accumulation of flavonoids found in plants not exposed to UV stress.
351 The increase of flavonoid accumulation in response to UV radiation was not
352 homogeneous across tissues: petals respond to UV by increasing anthocyanins, calyces and
353 leaves respond by increasing flavones, and stems through both anthocyanin and flavones.
354 This result is not surprising because the biosynthesis of flavonoids is tissue-specific regulated
355 [64]. The depletion of anthocyanins in petals en el UV-absent treatment is translated in a
356 change color intensity [65], which may be differentially perceived by the insect pollinators
357 [66]. On the other hand, calyces of plants from both UV-exclusion and UV-present
358 treatments of Sines population showed higher levels of flavones than those of Furnas in each
359 treatment. This difference may reflect a local adaptation of flavones biosynthesis to the
360 higher UV radiation of the Sines population compared with Furnas [39]. However, further

361 studies are necessary to assess whether flavonoid biosynthesis in *S. littorea* shows signals of
362 local adaptation across the UV radiation gradient in the distribution area.
363

364 Effects of UV radiation on photosynthetic performance

365 *Silene littorea* showed a higher decline of the quantum efficiency of PSII when plants
366 were exposed to UV stress, especially in leaves. Many authors have showed that the UV part
367 of sunlight is potentially highly important in photoinhibition of PSII of leaves. For example,
368 *Cucurbita pepo* leaves under UV stress exhibit a parallel decrease in photosynthetic activity
369 [42]. In addition, Albert et al. [67] showed that the PSII performance and net photosynthesis
370 of *Salix arctica*, is negatively affected by the ambient solar UV-B radiation. Given that *S.*
371 *littorea* was more susceptible to photoinhibition when it is exposed to UV stress, our
372 findings add evidences that the ambient solar UV radiation is a significant stress factor for
373 the photosynthetic activity of plants.
374 Despite the negative effects of UV stress on the photosynthetic activity in *S. littorea*,
375 this species seems to have an optimal light-stress recovery system and does not incur in
376 chronic photoinhibition, expressed as *Fv/Fm* values within the range of healthy plants after
377 relaxation of photoinactivation. In plants, when incident light surpasses the energy
378 assimilated by the photosynthetic apparatus, the excessive energy causes photoinhibition
379 and the formation of ROS, which results in photo-oxidative damage and an eventual decline
380 in photosynthetic activity [15,28,68]. The photoprotection mechanism of plants involves a
381 variety of defense agents against light-induced ROS, including the synthesis of antioxidant
382 anthocyanins and flavonoids [12,13]. In this regard, dihydroxy B-ring-substituted flavonoids
383 located in the chloroplasts help antioxidant enzymes to reduce light-induced ROS and those

384 diffusing out of the chloroplast are scavenged by vacuolar flavonoids [15]. In addition, leaves
385 accumulating anthocyanins incur in less photoinhibition after a saturating light stress as
386 compared with green leaves [27,69]. We hypothesized that flavonoids (both anthocyanins
387 and flavones) of *S. littorea* may contribute to photoprotection to thrive in habitats with
388 highly solar radiation such as coastal foredunes along the Iberian Peninsula [39]. Thus,
389 flavonoid biosynthesis may be of particular benefit to *S. littorea* to prevent photoinhibition
390 in this light-stressed habitat, as it was found in *Silene germana* [70].
391

392 Effects of UV radiation on reproductive output

393 Plants exposed to UV produced approximately three times less total number of seeds
394 per plant than those shielded from UV, driven primarily by a decrease in total flower
395 production. In a previous study, we found that flower production in *S. littorea* increases as a
396 consequence of high natural sunlight levels [71], but exposure to sunlight also entails the
397 exposure to harmful UV wavelengths. Here, we demonstrated that the absence of these
398 harmful effects in the UV-exclusion treatment allows the absorption of PAR and enhances
399 flower production. Although many studies often report enhanced flowering when plants
400 were exposed to supplemental UV radiation (e.g. [72,73]), other studies have reported the
401 opposite effect. For example, Feldheim and Conner [74] reported that supplemental UV-B
402 radiation was generally detrimental to flowering in *Brassica nigra* and *B. rapa*, while plants
403 from a lowland population of *Silene vulgaris* increase their flower production in the absence
404 of UV-B [75].
405 Additionally, we found that the proportion of flowers yielding fruits was nearly
406 double in plants under UV stress. Even though other studies have reported increasing

fecundity in plants exposed to moderate supplemental UV radiation [76], we suggest that significant differences in fruit set between light treatments could be influenced by the resources allocated to the high flower production of plants growing in the absence of UV stress. In addition, pollinators can become saturated at high flower densities [77,78], resulting in a decrease of per-flower visitation. Thus, despite the fact that absolute fruit production was almost four times higher in plants shielded from UV light, the elevated number of flowers of these plants not visited by pollinator may have led to a reduced fruit set. Experimental plants were fully accessible by pollinators around the study area (mostly hymenopterans), thus we can rule out that any architectural effect of the experiment might difficult pollinator visits.

Pollen production decreased in plants exposed to UV light is consistent with results in other species [36,37,79]. Since male gametes of plants are encased in pollen grains, decreasing pollen production is expected to have an adverse impact on male fitness of *S. littorea*. Conversely, ovule production was similar in plants from both light treatments. Ovules occur in ovaries, which are well protected against UV stress due to their accumulation of UV-absorbing compounds that attenuate UV radiation [7,33]. In *S. littorea*, upper anthers occur slightly beyond the corolla opening at anthesis and are more exposed to UV radiation, whereas carpophore is embedded in the calyx. Thus, ovule production is less likely to be compromised by solar radiation since ovules are protected from UV radiation by several layers of tissue.

Conclusions

UV radiation incurred a trade-off between flavonoid protection and reproduction in *S. littorea*. We propose that flavonoid production was activated as a defense mechanism against UV radiation, presumably because of their antioxidant nature, which may prevent the chronic photoinhibition and promote a rapid photosynthetic recovery. Conversely, exposure to UV radiation negatively affected flower and pollen production in this species. This balancing between protection and reproduction may be beneficial to successfully survive in exposed coastal foredunes. Thus, the allocation of metabolic resources may provide an efficient photoprotective toolkit and, at the same time, guarantee the reproduction of this species in Mediterranean climates subjected to strong UV radiation.

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Supporting information

S1 Table. Number of plants for each maternal genotype, population and treatment (UV-present and UV-exclusion treatments). The number of plants sampled for anthocyanin and flavone concentration is indicated in parentheses.

S2 Table. Pearson correlation coefficients for the comparison between flavonoid production (anthocyanins and flavones) in each plant tissue and reproductive outputs of *S. littorea*.

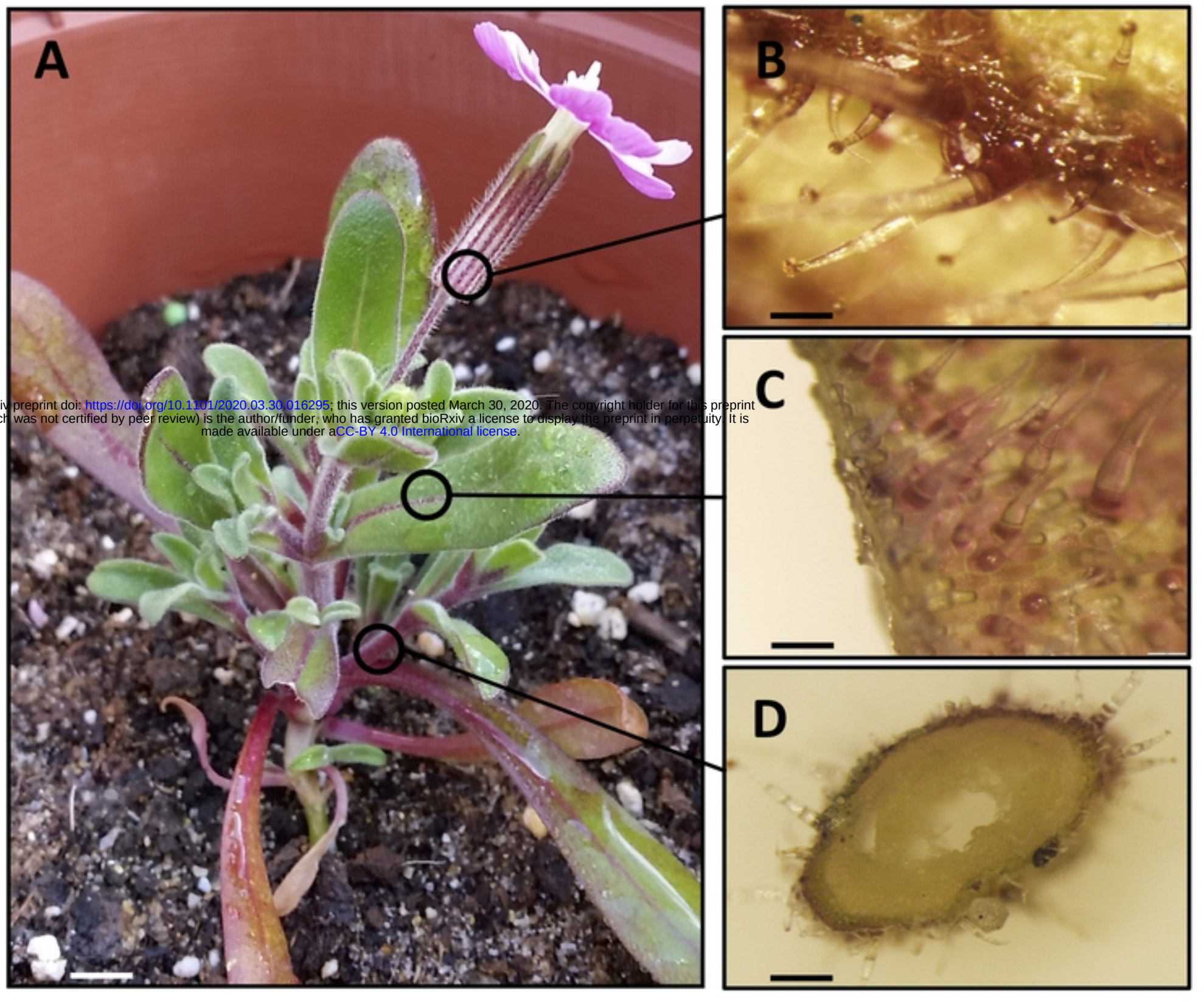

Figure1

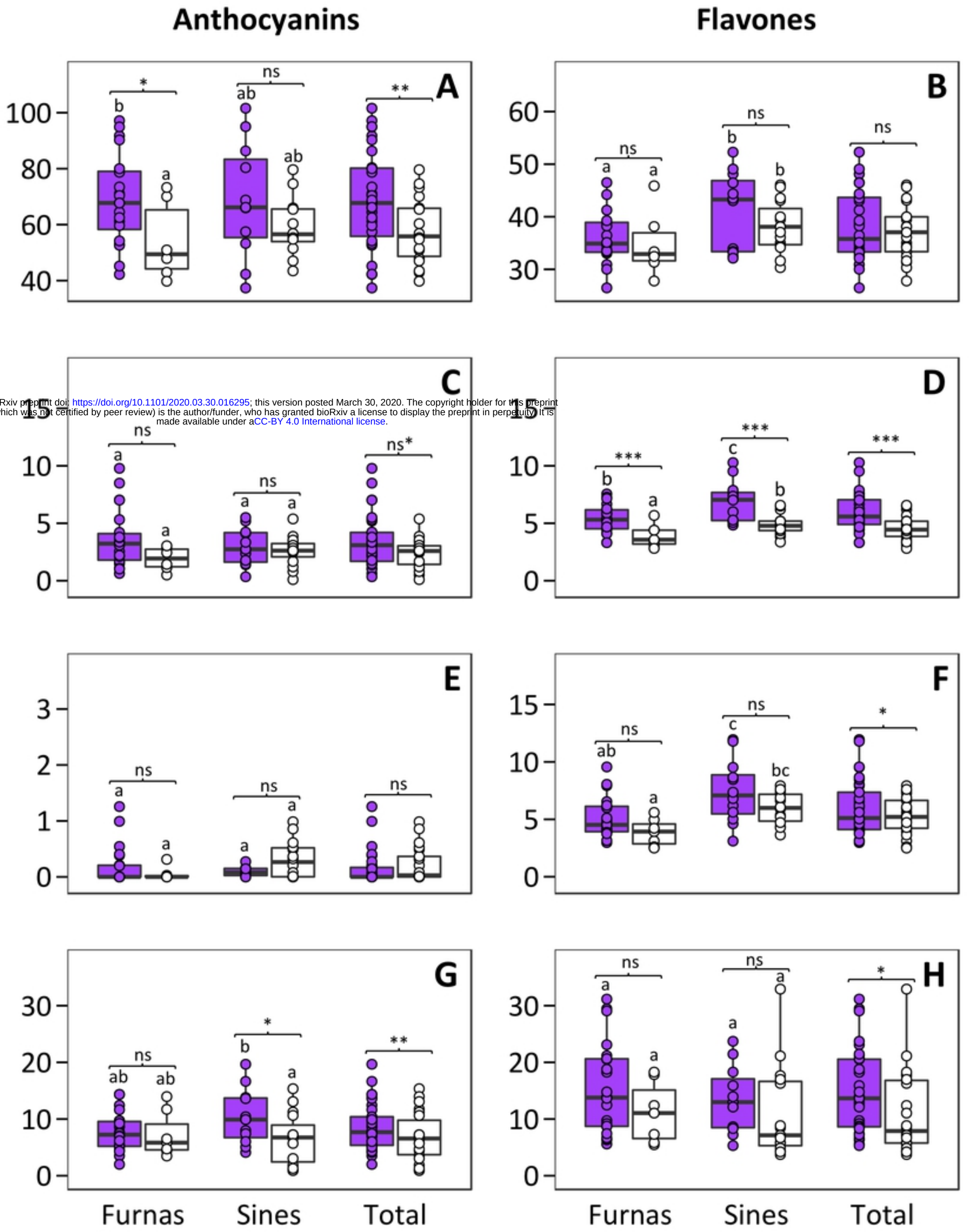

Figure2

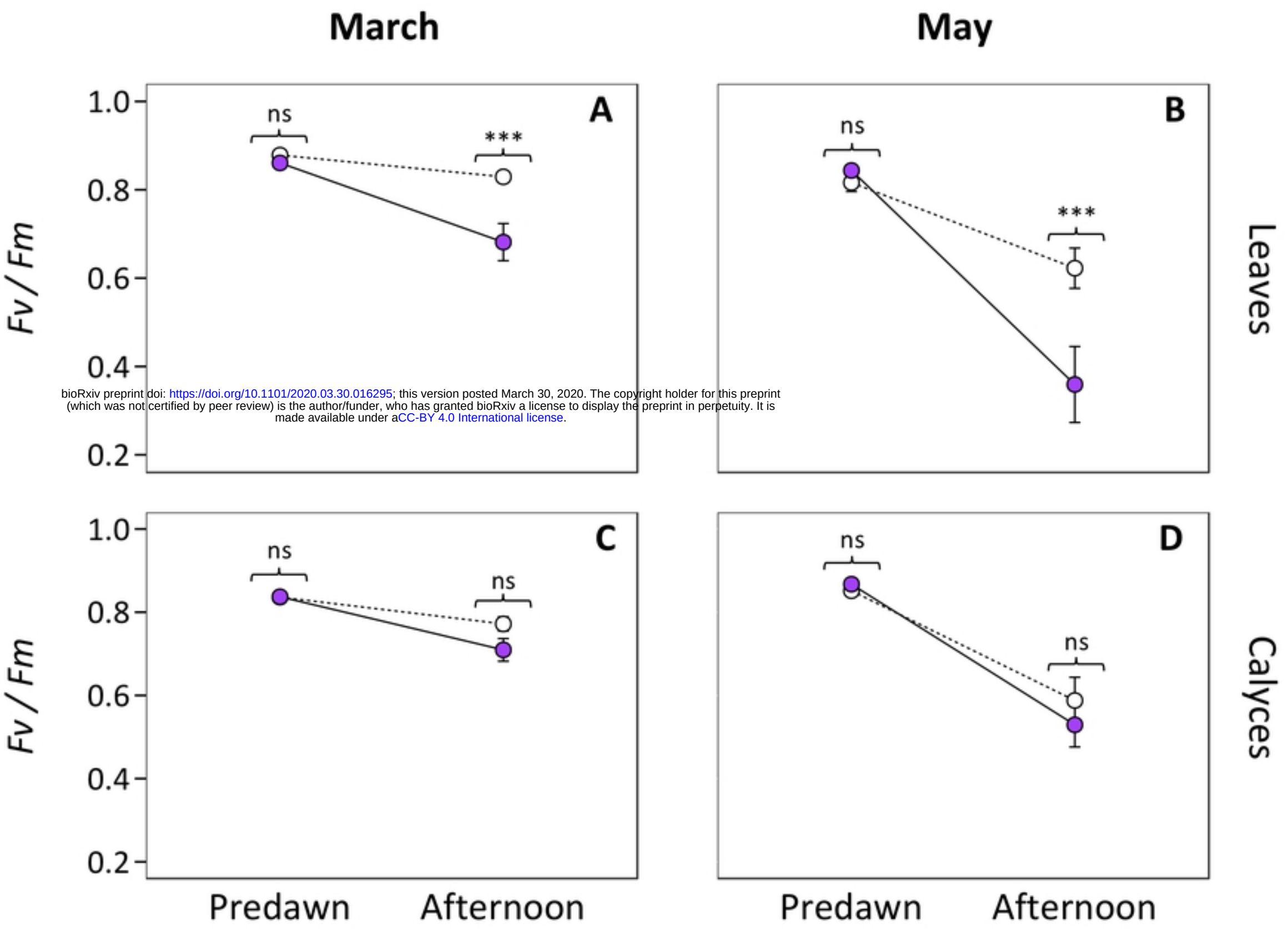

Figure3

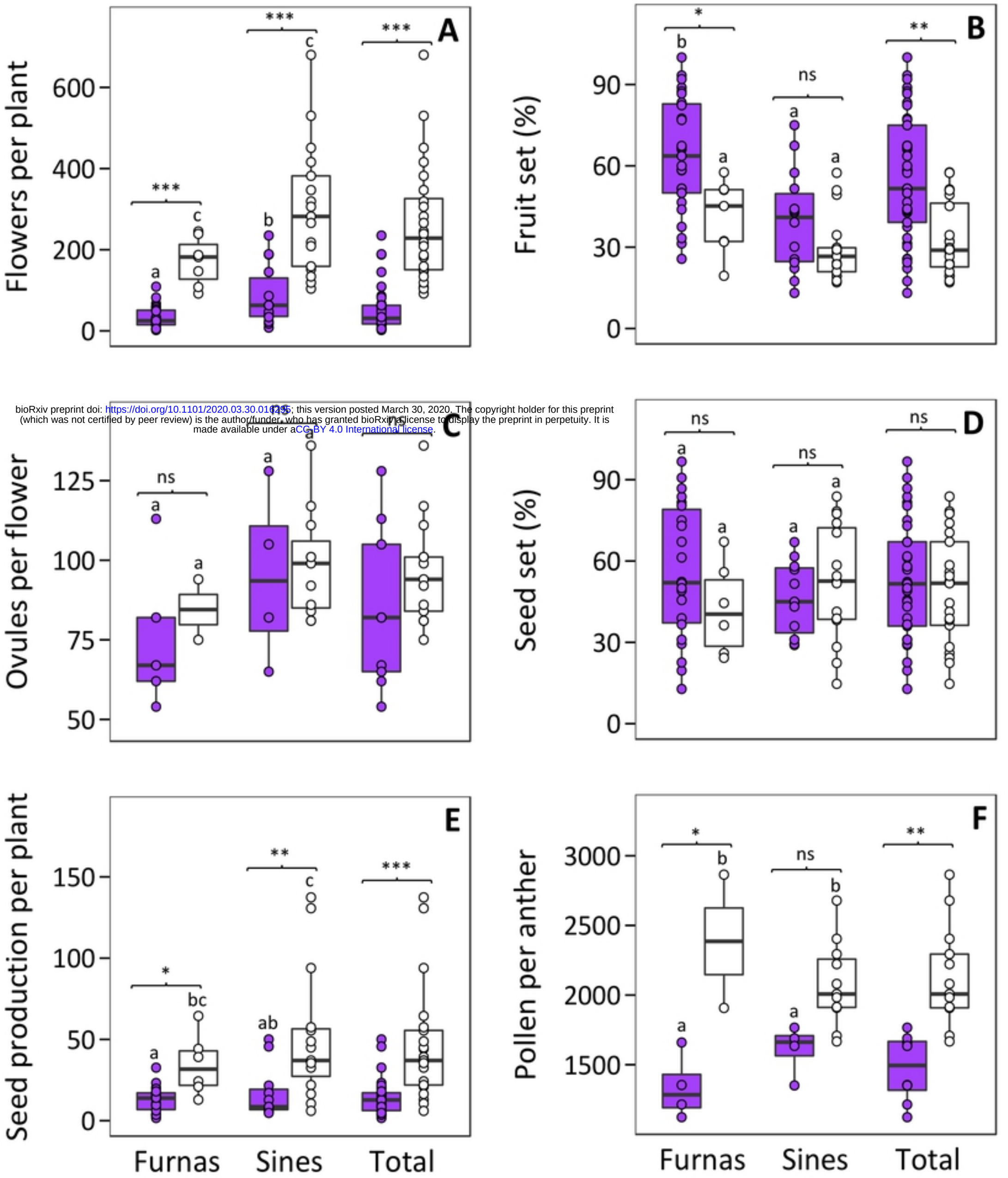

Figure4