Predicting the Responses of Functional Leaf Traits to Global Warming: An *in situ* Temperature Manipulation Design Using *Iris pumila* L.

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Article

Predicting the Responses of Functional Leaf Traits to Global Warming: An *in situ* Temperature Manipulation Design Using *Iris pumila* L.

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Abstract: Phenotypic plasticity is widely acknowledged as one of the most common solutions for coping with novel environmental conditions following climate change. However, it is less known whether current amounts of trait plasticity, which is sufficient for matching with contemporary climate, will be adequate when global temperatures exceed historical levels. We addressed this issue by exploring the responses of functional and structural leaf traits in *Iris pumila* clonal individuals to experimentally increased temperature (~1.5°C), using an open top chamber (OTC) design. We determined phenotypic values of specific leaf area, leaf dry matter content, specific leaf water content and leaf thickness in leaves sampled from the same clone inside and outside of the OTC deployed on it, over seasons and years within two natural populations. We analyzed the data by a repeated multivariate analysis of variance, which is primarily focused on profiles (reaction norms, RNs) of a variable gathered from the same individual at several different time points. We found that the mean RNs of all analyzed traits were parallel regardless of experienced temperatures, but differed in the level and the shape. The populations RNs were similar as well. Since the amount of plasticity in analyzed leaf trait was adequate for coping with elevated temperatures inside the OTCs, we predict that it will be also sufficient for responding to increased temperatures if they exceed the 1.5°C target.

Keywords: global warming; open-top chamber; functional leaf traits; SLA; LDMC; SLWC; LT; *Iris pumila* L.

1. Introduction

The current century has witnessed climate warming on an unprecedented scale. The Earth's global surface temperature in 2022 was 0.89 °C warmer than the twentieth century average, and 1.09 °C warmer than in 1880, when modern recordkeeping began [1–3]. There is general consensus that increasing emissions of greenhouse gases from human activities, notably carbon dioxide and methane, are causing a rapid global surface temperature increase worldwide [1–3]. This small, but significant long-term rise in the accumulated heat has altered a wide range of climate variables (e.g., weather and climate extremes) in every region of the earth [1–3]. Latest climate models predict that, without a sharp decline in greenhouse gas emissions by 2030, global warming will surpass 1.5°C in the following decades, threatening extinctions for a large number of species [4–6].

Anthropogenic climate warming has altered environmental conditions that plants experienced in their recent evolutionary history [7]. Given that Earth's atmosphere is heating up, one of the most pressing questions in the studies of plant adaptation to climate warming is how plant populations will respond to ongoing changes in habitat conditions [8–14]. Theoretically, the process of adaptation to the new environmental conditions in the original habitat, potentially occurs through two mechanisms: phenotypic plasticity [15] - the ability of single genotypes to produce the phenotypes

that match the climate they encounter [7,10,16–19], and genetic evolution - the process by which individuals with genes determining traits suitable for the new environmental conditions increase in frequency over generations [20–24]. In spite of growing evidence on the eco-evolutionary consequences of climate change, our understanding of how phenotypic plasticity and genetic evolution (inter)act to facilitate population persistence following climate warming is still limited [4,9–12,24].

Phenotypic plasticity is frequently assumed to be an important mechanism by which plants can cope with novel environmental conditions as those caused by global warming [8,11,25,26]. Phenotypic plasticity allows a population to respond quickly to the environmental variation by producing better matching phenotypes within a single generation. Hence, plasticity would be "a first stage of developmental rescue, prior to subsequent adaptation and evolutionary rescue of a population" [27]. Using individual-based simulation, Scheiner et al. [10] have recently found that the induction of phenotypic responses that move trait values (even partly) toward a new adaptive peak in a linearly changing environment can potentially keep pace with rapid environmental changes, averting population extinction. However, in an environment with accelerating change, the magnitude of plasticity needs to be much greater or standing genetic variation much higher to prevent extinction risk relative to linear environmental change with the same mean rate of change [12]. Using digital organisms, that is, self-replicating computer programs, Lalejini et al. [28] have found that adaptive plasticity allows plastic population to efficiently preserve more new adaptive traits in fluctuating environments compared to their non-plastic counterparts.

Both intra-annual (seasonal) and inter-annual climatic variation can promote increased phenotypic plasticity in plants [29–31]. While seasonal variation can select greater plastic responses in reversible physiological traits, inter-annual climatic variation can drive higher plasticity in plant size and allocation [17,32]. Results of a meta-analysis of global data on phenotypic plasticity have indicated that phenotypic plasticity for plant allocation was positively associated with climatic variation, whereas plasticity of other plant traits, including leaf morphology, physiology and size, was positively correlated with the average annual temperature [13].

One of the research questions that are still unresolved, and has no scientifically proved answer is whether the existing level of plasticity in natural populations that appeared to be sufficient for coping with contemporary temperature conditions will be enough once global surface temperature exceed the historical level? Given that the climate of a region define different elements, including solar radiation, temperature, humidity, precipitation, atmospheric pressure, and wind, the answer to the posed question requires the usage of specific experimental approaches that allow separating the effect of air temperature on plant individuals and populations from the effects of other climatic elements, if possible in nature [11].

Among different techniques that have been applied for modulating air temperature in the field, an *in situ* passive experimental warming by means of open top chambers (OTCs) devices (Figure 1) appeared to be very appropriate [33–35], especially for disentangling "cause-and-effect" relationships between plant responses and climate change [36]. Passive warming chambers have been widely applied to assess impacts of climate warming on plant communities in Alpine, Arctic, and Antarctic regions [33,34,37–40], as well as in the Tibetan Plateau [41–43]. According to Elmendorf et al. [40], open-top designs for manipulating field temperature was "yield consistent estimates of the magnitude of response of plant communities to climate warming", and as such are "best suited for forecasting impacts over the coming decades".

Our overarching objective in this study was to assess whether plastic phenotypic responses in leaf functional traits induced by the mean daily temperature increase of ~ 1.5 °C *in situ* - the global climate target, provide a potential mechanism by which plants can match quickly to novel environmental conditions (i.e., plasticity rescue) [25] prior to subsequent adaptation (i.e., evolutionary rescue) [44]. To do so, we carried out a temperature manipulation experiment by OTCs in nature, using clonal individuals of a perennial monocot, *Iris pumila* L., which inhabits exposed dune sites in the Deliblato Sands, Serbia. Here we analyzed intra-annual (seasonal) and inter-annual variation in phenotypic expressions of four key leaf traits: specific leaf area (SLA), leaf dry matter

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content (LDMC), specific leaf water content (SLWC), and leaf thickness (LT) in leaves developed on the same clonal plants inside and outside of every OTCs.

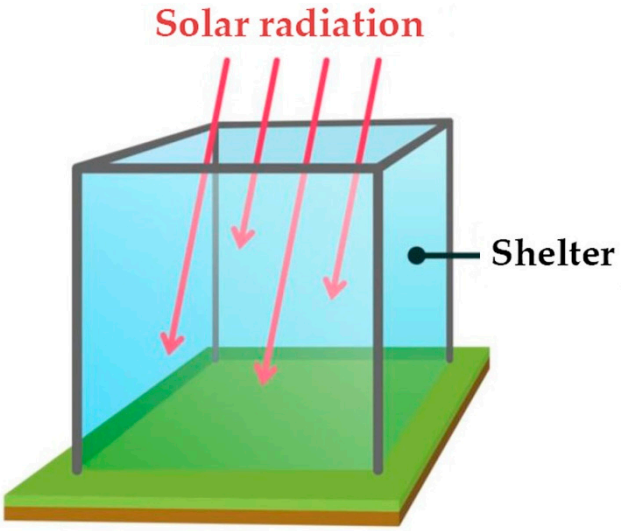

Figure 1. The scheme of an open top chamber (OTC) used in this study.

The leaf traits analyzed in this study are categorized as "functional traits" since they reflect various aspects of resource uptake and utilization [45]. Specific leaf area (SLA) (area/dry mass) is the major determinant of plant resource capture strategy [46]. SLA depends strongly on irradiation level [47], water supply [48] and nutrient availability [49,50]. Leaf dry matter content (LDMC) and leaf thickness (LT) are indicators of resource protection and conservation strategy [51,52]. LDMC correlates positively with leaf life-span and negatively with relative growth rate [53]. Leaf thickness (LT) affects the quantity of absorbed light and the speed of carbon dioxide diffusion [54]. LT is negatively associated with the rates of photosynthesis [55] and growth [56,57]. Because of that, LT has often been used as an indicator of ecological plant performance [58]. Specific leaf water content (SLWC) is commonly used as a predictor of plant whole-leaf photosynthesis and leaf area [59]. SLWC correlates well with nitrogen concentration and assimilation capacity [51], as well as with leaf tissue density and thickness [60].

An important prerequisite for making prediction about adaptiveness of phenotypes expressed in response to global warming is to characterize the shape of their reaction norms [61,62], i.e., the function that describes the dependence upon the environmental conditions of phenotypes produced by the same genotypes [63,64]. Assessment of variation in reaction norms is of pivotal importance for understanding how natural populations will respond to ongoing climate changes, as well as for predicting how non-genetic plastic responses can avert populations from extinction in a warming world.

The primary goal of this study was to compare plastic responses (reaction norms) of the four functional leaf traits: SLA, LDMC, SLWC and LT, expressed by the same clonal genotypes of *Iris pumila* under natural and experimentally elevated air temperatures in the field. We addressed the following questions: (1) What is the mean shape of plasticity (i.e., the species-specific reaction norm) in response to the grow temperature in *I. pumila*? (2) Does the magnitude and direction of the mean plastic responses to the growth temperature vary among leaf traits seasonally and/or annually? (3) Are the population-level reaction norms to variation in the growth temperature leaf-trait-specific?

2. Results

2.1. Abiotic environmental conditions

The climatic parameters, which illustrate clonal micro-environmental conditions prevailing within sampling sites, are given in Table 1. The measurements of instantaneous air and soil

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temperature, as well as soil moisture were taken just before leaf sampling. The mean instantaneous air and soil temperatures differed between the alternative temperature environments. The mean air temperature was higher inside the OTCs, while the corresponding soil mean temperature was lower compared to that prevailing outside of the OTCs. A reversed trend was observed outside the OTCs: the mean air temperature was lower, but the mean soil temperature was higher relative to that recorded inside the OTCs. Both *I. pumila* populations exhibited similar temperature trends over the entire experimental period, i.e., throughout the two seasons, spring and summer and two consecutive years, 2018 and 2019. In contrast to that, the mean soil moisture (expressed in percentage) appeared to be relatively constant over the whole clonal individuals, regardless of the ambient temperature they experienced. Seasonal variation in the air temperatures, both inside and outside the OTCs showed a contrasting trend between the two successive years. In 2018, the mean temperatures have greater values in the spring compared to the summer. However, in 2019, the summer mean air temperatures were greater than the spring air temperature means. Soil moisture showed an opposite trend; the mean soil moisture in spring 2018 was lower than in spring 2019, whereas in summer 2019, the mean soil moisture was higher relative to that recorded in summer 2018. Considering the mean soil temperature, in both years, the summer mean values were constantly higher than those recorded in the spring. Thus, the climate conditions prevailing in Deliblato Sands during 2018 and 2019 characterized an exceptionally dry and hot spring and rainy summer in 2018, followed by a drizzly spring and hot, dry summer in 2019. Similarly to instantaneous air temperatures, the mean air temperatures recorded by data loggers were consistently higher inside the OTCs compared to the natural ones prevailing outside OTCs (Table 1).

Table 1. The climatic variables (mean ± SE) recorded outside and inside the OTCs, over the two seasons (spring and summer) and two successive years (2018 and 2019) in two natural populations of Iris pumila (Population 1 and Population 2) settled in the Deliblato Sands. Statistical significance (Student's t-test) of the differences: ns- non significant, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Climatic variable	Year 1						Year 2					
	Spring			Summer			Spring			Summer		
	Outside	Inside	*p*	Outside	Inside	*p*	Outside	Inside	*p*	Outside	Inside	*p*
Instantaneous air temperature T_I (^{0}C)												
Population 1	32.3 ± 0.3	34.3 ± 0.3	****	29.4 ± 0.1	29.7 ± 0.2	ns	28.2 ± 0.2	28.5 ± 0.2	ns	32.2 ± 0.3	33.5 ± 0.3	****
Population 2	31.7 ± 0.4	34.1 ± 0.4	****	31.4 ± 0.4	32.3 ± 0.4	***	26.7 ± 0.2	27.1 ± 0.2	ns	31.5 ± 0.3	32.5 ± 0.4	****
Grand mean	32.0 ± 0.4	34.2 ± 0.4	****	30.4 ± 0.3	31.0 ± 0.3	***	27.4 ± 0.2	27.8 ± 0.2	*	31.9 ± 0.3	33.0 ± 0.4	****
Logged air temperature T_L (^{0}C)												
Population 1	20.0 ± 0.1	21.4 ± 0.2	***	26.9 ± 0.2	27.7 ± 0.2	****	23.9 ± 0.1	24.7 ± 0.1	****	26.4 ± 0.1	28.5 ± 0.2	***
Population 2	19.9 ± 0.1	21.0 ± 0.1	***	27.0 ± 0.2	29.4 ± 0.2	**	20.7 ± 0.2	23.0 ± 0.5	**	28.1 ± 0.2	29.4 ± 0.4	**
Grand mean	20.0 ± 0.1	21.2 ± 0.2	***	27.0 ± 0.2	28.5 ± 0.2	***	22.3 ± 0.2	23.8 ± 0.3	***	27.2 ± 0.2	29.0 ± 0.3	***
Instantaneous soil temperature (^{0}C)												
Population 1	18.0 ± 0.2	17.5 ± 0.2	*	23.5 ± 0.3	23.0 ± 0.2	**	13.8 ± 0.3	13.6 ± 0.3	ns	24.7 ± 0.3	24.8 ± 0.3	ns
Population 2	20.6 ± 0.4	19.3 ± 0.3	****	24.1 ± 0.3	23.7 ± 0.3	*	14.1 ± 0.4	12.9 ± 0.4	****	24.1 ± 0.4	23.8 ± 0.3	ns
Grand mean	19.3 ± 0.3	18.4 ± 0.3	****	23.8 ± 0.3	23.4 ± 0.3	***	14.0 ± 0.4	13.3 ± 0.4	***	24.4 ± 0.4	24.3 ± 0.3	ns
Instantaneous soil moisture (%)												

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Population 1	5.0 ± 0.1	4.8 ± 0.1	ns	9.2 ± 0.4	9.2 ± 0.4	ns	8.5 ± 0.3	8.6 ± 0.3	ns	4.6 ± 0.1	4.6 ± 0.1 ns
Population 2	5.3 ± 0.3	5.4 ± 0.3	ns	7.4 ± 0.3	7.0 ± 0.3	ns	8.8 ± 0.3	8.5 ± 0.3	ns	4.7 ± 0.2	4.8 ± 0.2 ns
Grand mean	5.2 ± 0.2	5.1 ± 0.2	ns	8.3 ± 0.4	8.1 ± 0.4	ns	8.6 ± 0.3	8.6 ± 0.3	ns	4.6 ± 0.2	4.7 ± 0.2 ns

2.2. Phenotypic responses of SLA, LDMC, SLWC and LT to temperature

To examine the phenotypic responses of leaf functional traits to temperature, the values of SLA, LDMC, SLWC, and LT were measured over time on the same clonal individuals of I. pumila occupying two sun-exposed natural populations. Leaves were sampled inside and outside the OTCs once per each season (spring and summer), during a two-year period. The mean values and corresponding standard errors for SLA, LDMC, SLWC, and LT are presented in Table 2. As can be seen, the phenotypic values of all examined functional leaf traits changed with air temperature. In both populations, throughout the whole vegetation season, the mean SLA values were higher (up to 9 %) in ramets developed inside an OTC, at experimentally elevated temperature, compared to that developed outside the same OTC. An inverse pattern was seen for LDMC, SLWC and LT, which appeared to be lower in leaves experiencing elevated air temperatures than those grown under ambient temperature conditions. All the changes described were similar during both experimental years (Tables 2 and 3).

Table 2. Means and standard errors (SE) for the four functional leaf traits: specific leaf area (SLA, in $cm^2\ g^{-1}$), leaf dry matter content (LDMC, in $g\ g^{-1}$), specific leaf water content (SLWC, in $g\ cm^{-2}$) and leaf thickness (LT), in Iris pumila clonal individuals from two natural populations (Population 1, n=24 and Population 2, n=21) during spring and summer of 2018 (Year 1) and 2019 (Year 2), expressed outside and inside of the OTCs in the Deliblato Sands.

	Year 1								**Year 2**							
Leaf trait	**Spring** SummerY1				**Summer**				**Spring** SummerY1				**Summer** SummerY2			
	Outside OTC		**Inside OTC**		**Outside OTC**		**Inside OTC**		**Outside OTC**		**Inside OTC**		**Outside OTC**		**Inside OTC**	
Population 1	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
SLA	164.97	2.65	177.78	2.36	151.03	1.85	157.75	1.84	144.12	1.79	155.52	2.43	157.56	2.46	178.83	2.49
LDMC	0.156	0.001	0.151	0.002	0.223	0.003	0.222	0.002	0.205	0.002	0.202	0.002	0.193	0.003	0.183	0.003
SLWC	0.033	0.001	0.032	0.001	0.024	0.001	0.022	0.001	0.027	0.001	0.026	0.001	0.026	0.001	0.026	0.001
LT	0.039	0.001	0.037	0.001	0.030	0.001	0.028	0.001	0.034	0.001	0.032	0.001	0.032	0.001	0.031	0.001
Population 2																
SLA	176.30	4.22	186.06	2.70	152.17	3.30	163.25	3.14	144.60	2.62	160.23	2.79	166.87	3.19	168.60	3.58
LDMC	0.157	0.002	0.157	0.002	0.231	0.003	0.228	0.002	0.195	0.002	0.190	0.003	0.191	0.003	0.186	0.003
SLWC	0.030	0.001	0.029	0.001	0.022	0.001	0.021	0.001	0.029	0.001	0.026	0.001	0.026	0.001	0.025	0.001
LT	0.036	0.001	0.034	0.002	0.028	0.001	0.027	0.001	0.036	0.001	0.032	0.001	0.032	0.001	0.031	0.001
Pooled Populations																
SLA	170.26	2.54	181.64	2.14	151.56	1.81	160.32	1.79	144.34	1.53	157.72	1.85	161.92	2.08	174.06	2.25
LDMC	0.157	0.001	0.154	0.001	0.227	0.002	0.225	0.002	0.200	0.002	0.196	0.002	0.192	0.002	0.184	0.002
SLWC	0.032	0.001	0.030	0.001	0.023	0.001	0.022	0.001	0.028	0.001	0.026	0.001	0.026	0.001	0.026	0.001
LT	0.037	0.001	0.036	0.001	0.029	0.001	0.028	0.001	0.035	0.002	0.032	0.001	0.032	0.001	0.031	0.001

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Table 3. Results of a repeated-measures analysis of variance (profile analysis) on the four functional leaf traits: specific leaf area (SLA, in $cm^2\ g^{-1}$), leaf dry matter content (LDMC, in $g\ g^{-1}$), specific leaf water content (SLWC, in $g\ cm^{-2}$) and leaf thickness (LT), in *Iris pumila* clonal individuals from two natural populations over two seasons (spring and summer) and two years (2018 and 2019), expressed outside and inside the OTCs in the Deliblato Sands. Wilks' λ is presented for the multivariate analyses because the F-test corresponding to this statistic is exact.

A. Between-subjects	SLA			LDMC			SLWC			LT		
Source of variation	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Population (P)	1	3.73	.0569	1	0.00	.9539	1	7.84	.0063	1	7.10	.0092
Treatment (T)	1	32.70	.0001	1	8.02	.0058	1	12.42	.0007	1	15.44	.0002
P x T	1	0.78	.3786	1	0.08	.7819	1	0.33	.5680	1	0.15	.6966
Error	86			86			86			86		

B. Within-subject

Source of variation	Wilks' λ	*F*	df	*p*	Wilks'λ	*F*	df	*p*	Wilks'λ	*F*	df	*p*	Wilks'λ	*F*	df	*p*
Year (Y)	0.6749	41.43	1,86	.0001	0.9550	4.05	1,86	.0474	0.9999	0.00	1,86	.9927	0.9986	0.12	1,86	.7338
Season (S)	0.9761	2.05	1,86	.1558	0.1180	643.01	1,86	.0001	0.1543	471.19	1,86	.0001	0.2034	336.86	1,86	.0001
Y x S	0.2075	328.41	1,86	.0001	0.0609	1326.2	1,86	.0001	0.2506	257.17	1,86	.0001	0.3792	140.88	1,86	.0001
P x Y	0.9236	7.12	1,86	.0091	0.7980	21.77	1,86	.0001	0.6906	38.52	1,86	.0001	0.6831	39.90	1,86	.0001
P x S	0.9546	4.09	1,86	.0462	0.8939	10.21	1,86	.0020	0.9996	0.04	1,86	.8516	0.9996	0.04	1,86	.8508
P x Y x S	0.9919	0.70	1,86	.4046	0.9597	3.61	1,86	.0607	0.9562	3.94	1,86	.0504	0.9507	4.46	1,86	.0377
T x Y	0.9843	1.38	1,86	.2442	0.9726	2.42	1,86	.1234	0.9903	0.85	1,86	.3603	0.9910	0.78	1,86	.3785
T x S	0.9900	0.87	1,86	.3538	0.9972	0.24	1,86	.6266	0.9812	1.64	1,86	.2031	0.9585	3.73	1,86	.0569
T x Y x S	0.9999	0.01	1,86	.9290	0.9860	1.22	1,86	.2724	0.9947	0.46	1,86	.5002	0.9710	2.57	1,86	.1124

2.3. Reaction norm graphs for SLA, LDMC, SLWC and LT

The mean reaction norms of SLA, LDMC, SLWC, and LT for leaves of I. pumila developed inside and outside the OTCs are depicted in Figure 2. Reaction norms of most leaf traits were parallel over the whole research interval. The only exception was LT, which had parallel reaction norms between the time points Y1S1 and Y1S2, and convergent ones at the time point Y2S2 (Figure 2). The level of reaction norms was trait-specific, as well. The mean reaction norms of SLA ranked higher at elevated temperatures (inside the OTCs) than at ambient temperature, in contrast to LDMC, SLWC and LT which had greater levels outside the OTCs than inside of them (Figure 2). The variation in the shape and the level of reaction norms appeared to be trait- and time-interval specific ((Figure 2). For example, the levels of reaction norms for SLA, SLWC, and LT were decreasing between spring and summer of the first year (time points Y1S1 and Y1S2), but increasing in the second year (time points Y2S1 and Y2S2) for SLA, and decreasing for SLWC and LT. LDMC was the only leaf trait, which reaction norms exhibited increasing levels between time points Y1S1 and Y1S2, but a slightly decreasing levels between time points Y2S1 and Y2S2. Interestingly, the shape of reaction norms over time changed from concave for SLA to convex for LDMC to zig-zag for SLWC and LT at both temperature regimes (Figure 2).

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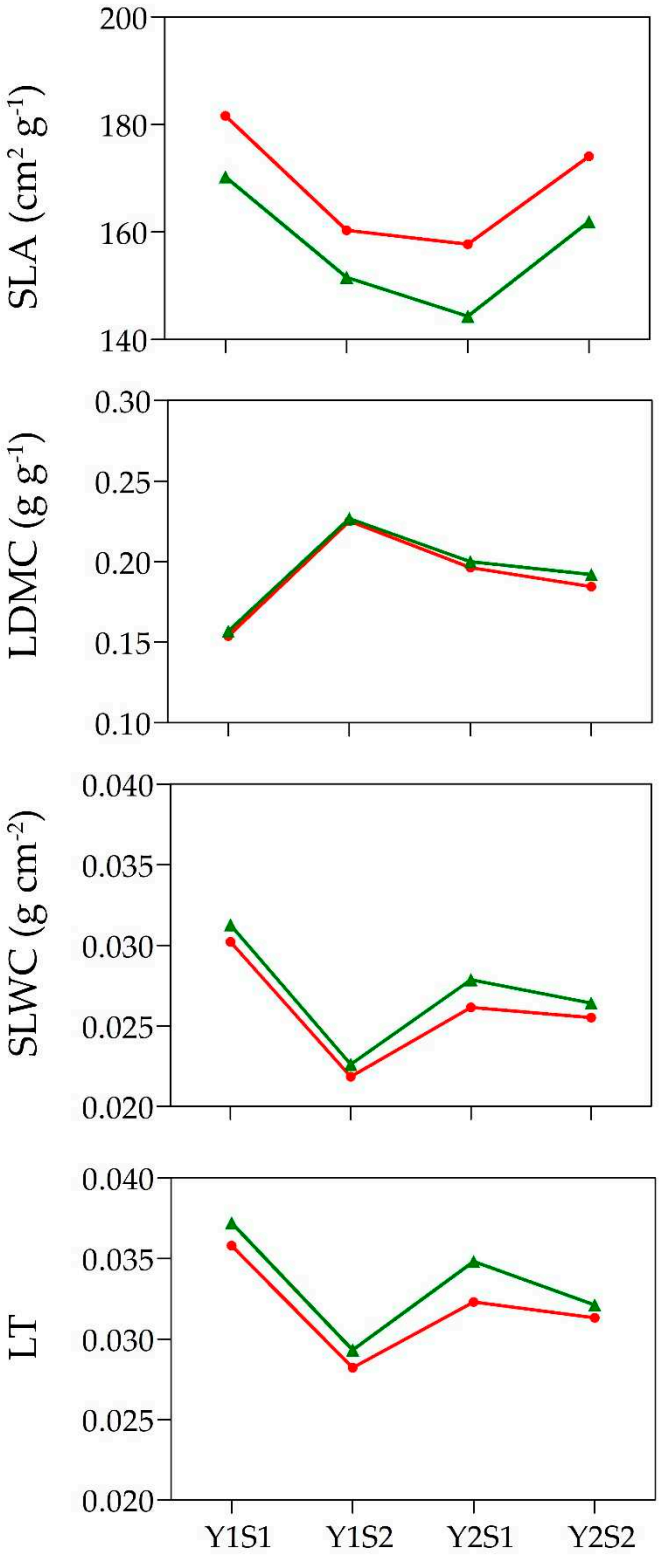

Figure 2. Reaction norm plots for the mean values of specific leaf area (SLA), specific leaf water content (SLWC), leaf dry matter content (LDMC) and leaf thickness (LT) of *Iris pumila* clonal individuals from two natural populations (Population 1, n = 24 and Population 2, n = 21), during spring (S1) and summer (S2) 2018 (Year 1) and 2019 (Year 2), expressed outside (green line, triangle symbol) and inside (red line, circle symbol) of the OTCs in the Deliblato Sands.

2.4. Profile analysis

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To examine whether there is a significant difference between the mean response profiles of plants grown under different temperature conditions we applied a profile analysis on functional leaf traits: SLA, LDMC, LWC and LT. The results of profile analyses are presented in Table 3. Because this procedure consists of three tests: the test of parallelism, the test of level, and the test of flatness, and since these test should be carried out in a sequential order, we get started with testing whether the mean response curves are parallel. In terms of profile analysis, this is a test of whether there is a significant interaction of the within-subject main effects (year, season) with the temperature treatment. The profile analysis revealed that the probabilities of F-statistics ($p > F$) of all interaction effects (treatment-by-season, treatment-by-year, and treatment-by-year-by-season) were statistically insignificant at $\alpha = 0.05$ (Table 3). A lack of significant interactions between the within-subject main effects and the temperature treatment corroborated the null hypothesis that the mean response profiles of SLA, LDMC, SLWC and LT between plants grown inside the OTCs and plants grown outside the OTCs were parallel over time.

Given that the mean response profiles of the two groups were parallel for each trait under study, we tested whether the profiles were at equal levels (i.e., there was no a group difference). Profile analysis revealed that the treatment effect for all four leaf traits was statistically significant (SLA, $p < 0.0001$; LDMC, $p = 0.0058$; SLWC, $p = 0.0007$; and LT, $p = 0.0002$; Table 3), indicating that the levels of the two groups were significantly different from one another. Based on this test, the hypothesis of equal levels was rejected.

Profile analysis of the between-population effects showed that the mean response profiles between populations for all analyzed traits were the same for SLA and LDMC (all $ps > 0.05$), but differed for SLWC and LT ($p = 0.0063$ and $p = 0.0092$, respectively). The results indicated that plants from both populations responded similarly to elevated temperatures, regarding SLA and LDMC.

The tests of flatness hypothesis are presented in Table 3. The statistically significant multivariate F-values for the season and year main effects suggested that following functional leaf traits elicited the same average response to temperature variation: SLWC and LT over years ($p = 0.7338$ and $p = 0.9927$, respectively) and SLA over seasons ($p = 0.1558$). All traits changed their response profiles either over seasons (LDMC, SLWC and LT; all $ps < 0.0001$) or across years (SLA and LDMC; $p = < 0.0001$, $p = 0.0474$, respectively).

Individual ANOVAs (F-tests) on each of the contrasts for the main within-subject factors, season and year, revealed that, when averaged over both treatments, there was a significant change in all analyzed leaf traits over time (a significant mean effect in all ANOVAs) (Table 4). A highly significant treatment effect ($p = 0.0001$) was obtained exclusively for LT, indicating that the mean response profile between the two groups trait differed only in the second year of repeated measurements (Table 4).

Table 4. Analyses of variance on each of the contrasts of the within-subject factor (seasons) for the level specific leaf area (SLA, in $cm^2\ g^{-1}$), leaf dry matter content (LDMC, in $g\ g^{-1}$), specific leaf water content (SLWC, in $g\ cm^{-2}$) and leaf thickness (LT), in *Iris pumila* clonal individuals from two natural populations, expressed outside and inside the OTCs in the Deliblato Sands.

Source of variation	SLA			LDMC			SLWC			LT		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Year1												
					Contrast variable: summer – spring							
Mean	1	162.03	.0001	1	2509.31	.0001	1	718.92	.0001	1	448.63	.0001
Treatment	1	0.70	.4054	1	0.27	.6045	1	0.13	.7195	1	0.08	.7784
Error	88			88			88			88		
Year2												
					Contrast variable: summer – spring							
Mean	1	100.70	.0001	1	26.17	.0001	1	234.75	.0001	1	27.43	.0001
Treatment	1	0.13	.7180	1	0.99	.3225	1	2.04	.1569	1	5.93	.0169
Error	88			88			88			88		

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Overall, our results suggested that the response profiles of functional leaf traits in clonal individuals of I. pumila growing inside and outside OTCs were parallel, but not coincidental and flat. This implies that an increase in air temperatures of about 1.5° C can evoke changes in both the level and shape of the mean response profiles of leaf traits in I. pumila compared to those expressed at ambient air temperatures.

2.5. Kendall rank correlations between functional leaf traits

The results of the Kendall rank correlation analyses revealed that functional leaf traits in I. pumila were mostly negatively correlated one to the others, with exception of LT, which was positively correlated exclusively with SLWC in both temperature treatments. The correlation between SLWC and LT was the highest compared to all other correlations (Tau b = 0.8673 and Tau b = 0.8834; both $p < 0.0001$, in the high and low temperature treatments, respectively) between functional leaf traits. Correlations between LT and LDMC were negative in sign and moderate in strength (Tau b = -0.559 and Tau b = -0.502, both $p < 0.0001$, in the high and low temperature treatments, respectively) as were correlations between SLWC and LDMC (Tau b = -0.623 and Tau b = -0.589, both $p < 0.0001$, in the high and low temperature treatments, respectively). The lowest negative correlations were detected between SLA and LDMC (Tau b = -0.389 and Tau b = -0.353, both $p < 0.0001$, in the high and low temperature treatments, respectively), and between SLA and LT in the low temperature treatment (Tau b = -0.145, $p < 0.0039$, in the low temperature treatment).

2.6. Regression analysis

To better understand the association between air temperature and leaf functional traits we employed a robust regression analysis. The results of the robust regression analyses have shown that the relationship between SLA and the mean daily temperature (Logged air temperature, T_L) was generally non-significant, with the exception of a marginally significant association with T_L in the summer ($b = 4.2954$, $p < 0.06$). LDMC exhibited a significantly positive relationship with T_L over the whole experiment ($b = 0.0050$, $p < 0.0001$). Conversely, the association between T_L and both SLWC and LT was significantly negative (for both traits $b = -0.0009$, $p < 0.0001$).

3. Discussion

The goal of this study was to get a broader insight into the phenotypic changes of functional leaf traits that may likely occur in response to global climate warming, as well as to assess whether these changes are sufficiently fast to keep pace with rapid shifts in the environment. To achieve that goal, we compared the average reaction norms of the four functional leaf traits, SLA, LDMC, SLWC, and LT, in *I. pumila* clonal individuals elicited in response to air temperatures prevailing inside and outside the OTCs over two successive vegetation seasons. Since "A reaction norm is a mirror that reflects environmental effects into phenotypes" [65] and because each clonal individual has simultaneously experienced both the natural and experimentally increased (~1.5 °C) air temperature, we were able to forecast the magnitude and the direction of plastic changes in functional leaf traits that might happen if global surface temperature approaches the 1.5 °C global goal.

Very recently, Arnold et al. [62] have pointed out that a precursor to a better understanding of how phenotypic plasticity can potentially facilitate population persistence in novel and rapidly changing environments is to characterize the shape of reaction norms. The mean shape of reaction norms for the analyzed leaf traits in *I. pumila* appeared to be markedly dissimilar across the same environmental range, but very alike when compared under natural and experimentally elevated air temperatures. The results of a MANOVAR analysis corroborated the graphical features of these reaction norms. The mean reaction norms of the four analyzed leaf traits expressed at alternative temperature regimes were parallel over time, but not coincidental and flat. According to the obtained results, it seems reasonable to believe that functional leaf traits of *I. pumila* have the capacity to respond plastically to temperature increase due to global change, as well as that the rate of these

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plastic changes can be very fast, occurring within a single vegetative generation of this clonal plant. However, it is difficult to anticipate whether the plasticity of functional leaf traits to rapid temperature increase (as occurs presently due to global change) will generate phenotypes that match well with novel environmental conditions. Snell-Rood et al. [25] have suggested that initial plastic responses to novel environmental conditions are in many cases maladaptive due to the absence of past selection in these environments. Since microclimatic environmental conditions experienced by individual *I. pumila* clones differed exclusively in air temperature prevailing inside and outside the OTCs and because there was no signature of negative phenotypic selection operating inside the OTCs (e.g., dead ramets), it is likely that phenotypic changes of leaf functional traits elicited in response to higher air temperature inside the OTCs can be classified as adaptive.

The leaf traits included in this study are components of the leaf economics spectrum (LES) [66], whose variation and covariation reflect an ecological trade-off in resource acquisition and utilization strategies of plants [46,58,67–69]. As such, these traits are expected to be especially affected by global climate change [70,71].

One of the most important leaf functional traits is specific leaf area (SLA). This trait reflects the efficiency of resource utilization strategies of plants [70,72]. It has been recognized that the most influential factor in determining the spatial variation in SLA on a large scale is solar radiation. On a short spatial scale, however, SLA usually increases with the increase of available resource [70]. In our temperature manipulation experiments *in situ*, reaction norms of SLA had a consistently higher level at an elevated temperature than at a natural one, regardless of season or year. However, when phenotypic responses of SLA to temperature variation were averaged over both treatments, the reaction norm shape of this leaf trait changed both over seasons and across years. This result is in agreement with the statement that SLA responds nonlinearly to environmental change [70]. SLA is the only leaf trait measured in this study that expressed greater phenotypic values inside than outside of the OTCs. This conclusively means that producing leaves with a larger surface area relative to mass can be advantageous in warmer environments because of a greater capacity for light interception, as well as a higher cooling efficiency through transpiration. In spite of numerous pieces of evidence that SLA increases with temperature [73–75], the relationship between SLA and temperature is not universally positive, but can vary among plant species, ecosystems, and environmental conditions [75]. For example, some studies provided evidence that SLA tended to decrease in response to elevated temperatures to withstand heat stress, either by reducing leaf expansion or increasing leaf thickness [77–79].

Leaf dry matter content (LDMC) is a component of the LES [66] that is frequently used as an indicator of the position of a given plant species in "a fundamental trade-off between a rapid assimilation and growth at one extreme, and efficient conservation of resources within well-protected tissues at the other" [80] (p. 955). Accordingly, species with faster growth rates will usually exhibit thinner leaves with low LDMC values, fast resource acquisition rates, and leaf turnover, while species with slower growth rates are characterized by thicker leaves with high LDMC values and slower rates of leaf turnover [81–83]. However, in the face of rapid climate change, phenotypic plasticity of the LES traits can additionally impact the rate of plant growth [75]. We found that the mean reaction norms of LDMC in *I. pumila* were parallel, but exhibited distinct levels - greater at lower air temperatures - outside the OTCs and lower at higher temperatures - inside the OTCs. In addition, a MANOVAR analysis revealed that the shape of the reaction norm for LDMC changed both seasonally and interannually as well. The relationship between LDMC and global mean temperature is thought to be complex, context- and species- or ecosystem-dependent [84–86]. Because both nutrient uptake and assimilation rates can be amplified at higher temperatures, a tendency for LDMC to decline with temperature increase could be attributed to a greater investment of leaf nitrogen in photosynthetic organs and, consequently, a higher leaf respiration and biomass production rates [87]. Warming can indirectly affect LDMC through its direct effect on water content in plant tissue. Specifically, high temperatures can increase evapotranspiration rates, leading to greater water loss from the leaves and, in turn, LDMC decline [88]. In the case of *I. pumila*, plastic changes in LDMC to experimentally increased temperatures, which simulated the global target of 1.5 °C, altered in a direction that will

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likely maintain fitness in that microenvironment. Hence, it seems reasonable to believe that the plasticity of LDMC to temperature variation is adaptive, and that its amount is adequate for keeping pace with global warming.

Specific leaf water content (SLWC) is an important component of the LES owing to its key role in leaf thermal regulation and carbon assimilation as well as its strong correlations with other functional leaf traits [89,90]. The changes in precipitation patterns and water availability associated with global warming can affect the overall water status of plants, influencing SLWC [90]. Since different plant species display diverse capacities to adjust to or tolerate changes in temperature and water availability [91] and because temperature and SLWC quantitatively correlate with other leaf traits, such as photosynthetic capacity and SLA (or LDMC), SLWC can be used as an indicator of species-specific adaptation to environmental conditions [90]. In *I. pumila*, the reaction norms of SLWC appeared to be parallel over time at different temperature regimes, but their level was higher at the lower ambient temperature than at the higher one, indicating a negative impact of high air temperatures on leaf water availability. In contrast to the previous reports that variation in LDMC and, thus, in SLWC are relative constants over seasons [92], our results strongly indicate that the shape of reaction norms for SLWC did change over seasons and across years. The observed temporal changes in the average plastic responses of SLWC in *I. pumila* can be attributed to the immediate alteration in microclimate conditions taking place within their natural habitats.

Leaf thickness (LT) is a quantitative trait related to leaf form. In the context of the LES, LT reflects a tradeoff between a rapid growth versus drought and heat tolerance. Thinner leaves have a higher surface-to-volume ratio, which enhances the leaf's ability to dissipate excess heat through increased convective cooling [93]. By reducing leaf thickness, plants enhance their heat tolerance and minimize the risk of overheating at high air temperatures [94]. Low leaf thickness indicates a reduced structural support and a potentially limited water storage capacity [95,96]. The reduction in LT can be viewed as an adaptive modification that mitigates heat stress and optimizes heat dissipation [97]. The relationship between LT and warming was found to vary depending on plant species, environmental conditions, and the magnitude and duration of warming [98]. We found that LT in *I. pumila*, tended toward lower values at higher air temperatures compared to its lower counterparts. Similarly to other leaf traits measured, the reaction norms of LT were parallel to each other, while their level was greater at lower than at higher air temperature. The shape of reaction norms for LT is very similar to that depicted for SLWC, regarding both the intraclonal temperature variation as well as the seasonal and interannual environmental changes. Thus, producing thinner leaves at elevated temperatures would be a rapid phenotypic response that would protect leaf tissue from overheating by increasing the heat dissipation rate owing to a larger leaf surface area.

The phenotypic correlations among the four functional leaf traits, components of the LES, were similar in strength, irrespective of the temperature treatment, reflecting a conservative plant strategy of *I. pumila* in terms of resource acquisition and allocation. Our results corroborate the view that the LES - although present in different plant forms - appeared to be greatly independent of climate conditions.

4. Materials and Methods

4.1. The study species

The dwarf bearded iris, *Iris pumila* L. (Iridaceae), is a rhizomatous perennial herb indigenous to the Eurasian Steppe belt. The species extends from Austria in the west, through central and southeastern Europe, to west Siberia in the east [99–106].

Iris pumila is the component of grassland steppe ecosystem (the order Festucetalia valesiacae; [107,108] distributed in the Deliblato Sands (44º90'23''N, 21º11'32''E) - a large continental sandy area, situated in the southeastern part of the Pannonia Plain, in Banat, Serbia [99,108]. Within that region, natural populations of *I. pumila* mostly inhabit sun-exposed sites on the crests and windward slopes of sand dunes. The species propagates from creeping rhizomes (modified stems) that branch laterally from the central "mother rhizome", producing the first order branches - the "primary segments".

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Primary segments continue to branch, resulting in secondary, tertiary, and higher order branches. The rhizome segments of *I. pumila* are approx. 2.5 cm long and tightly packed, creating round-shaped clones of variable sizes (30 – 120 cm in diameter), depending on clone age [109,110]. The blooming faze of *I. pumila* is in early spring. Flowers display a conspicuous within-population colour variation. Violet, blue and yellow colour categories are more frequent, but white coloured flowers can be found as well [111]. Flower colour variation in *I. pumila* results from accumulation of multiple pigment classes such as anthocyanins and carotenoids, and their relative content in petal tissue [112]. Because the biosynthesis of anthocyanins and carotenoids are genetically controlled by structural and regulatory genes [113,114], flower colour can serve as a reliable genetic marker for discrimination between district floral genotypes within a colour polymorphic populations [111].

4.2. Open top chambers and experimental design

To simulate global warming, we used a rectangular (50cm x 50cm and 50cm, L, W, H) open top chamber (OTC) design (Figure 3). The walls of OTCs were made of a diffused light thermal film based on ethylene vinyl acetate (Guarniflon S.p.a.,Treviso, Italy), which did not change the spectral composition of transmitted light, so that the ratio of red to far-red light (RFR) within the OTCs remained unaffected. The thermal film was attached to four metal pales that were positioned vertically in the corners of the OTC and buried approx. 20 cm in the soil. The chambers passively increased air temperatures at 25 cm height by 1.5°C above ambient levels, on average over an entire year.

Figure 3. The open-top chambers (OTCs) deployed on individual clones of *Iris pumila* naturally growing on a dune slope in the Deliblato Sands (upper picture). Perspective and aerial view of the OTC positioned over *I. pumila* clone (lower picture).

For this study we choose two sun-exposed populations of *I. pumila* occurring in the dune system of the Deliblato Sands. One of these populations (hereafter named Population 1) inhabited the slope of a dune, whereas the other population (Population 2) resided along a dune crest (top). The areal distance between these populations was about 4 km.

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In April 2017, during the blooming phase of *I. pumila*, we randomly selected 30 large clones (more than 60 cm in diameter) within each population and marked each of them with a wooden pole displaying clone ID. To protect the intermixing with alien clones after the blooming phase, we removed all flowering ramets differing in coloration from the focal clone that were growing in its vicinity of 30 cm. In May 2017, after the flowering phase has ended, we positioned the OTCs over one half of each selected clone, leaving the other half to experience natural environmental conditions (Figure 3).

4.3. Measuring environmental variables

Within each of the two populations, we recorded the air temperature inside and outside of the OTCs using a thermal data logger with two external sensors (ThermaD-ta® logger TB2F, Electronic Temperature Instruments Ltd, UK). One of the sensors was placed inside, whereas the other outside of the OTC. A total of three devices were employed per population. Temperature measurements were taken at 30-minute intervals during the whole experimental period.

To assess the micro-climate conditions within individual clones, we recorded the following environmental variables inside and outside of each OTC, just before leaf sampling: air temperature (15 cm above ground), soil moisture and soil temperature (at 5 cm in depth), light intensity (photosynthetically active radiation, PAR), and Red / Far-red ratio (RFR). Air and leaf lamina temperature were recorded with an infrared laser non-contact thermometer (Crop TRAK, Spectrum Technologies, Inc., Plainfield, USA); soil moisture with soil moisture sensor (ML3 ThetaProbe Delta-T Devices Ltd., UK), soil temperature with contact stab digital thermometer (SuperFast Thermapen, Electronic Temperature Instruments Ltd, UK), PAR with a point quantum sensor (LI-190SA, LI-COR, Inc., Lincoln, USA) and RFR with R : FR sensor (Skye SKR-110, Skye Instruments Ltd., Powys, UK).

4.4. Leaf sampling and leaf traits measuring

A fully developed leaf was harvested from each of six ramets: three-growing inside and other three outside of every OTC. Leaf sampling was done once in each of the two growing seasons: spring and summer, during a two-year period. After sampling, leaves were immediately packed in plastic bottles, sealed with parafilm, and transported under cold conditions to the laboratory for the further analyses.

The phenotypic values of specific leaf area (SLA), specific leaf water content (SLWC), leaf dry matter content (LDMC) and leaf thickness (LT) were determined using standardized protocols for plant functional trait measurements [115]. Briefly, leaf surface area was scanned as a digital image by an optical scanner (HP ScanJet 3800; DPI) and measured using image analysis software (Image J, 1.51j8). Thereafter, leaf samples were oven-dried to a constant mass at 60°C for 72 h and their dry biomass weighed immediately after cooling down. SLA (in $cm^2\ g^{-1}$) was calculated as the projected area of a fresh leaf divided by its dry biomass. LDMC (in $g\ g^{-1}$) was computed as the ratio of leaf dry to fresh biomass. SLWC (in $g\ cm^{-2}$) was determined as the difference between fresh and dry leaf biomass divided by leaf area. LT was calculated according to the formula: 1 / SLA x LDMC [52].

4.5. Statistical analyses

Because half of the experimental clones experienced manipulated air temperatures, while the other half faced ambient temperature conditions, and since we measured repeatedly several functional leaf traits on the same clonal plant at different time points (i.e., in spring and summer of two successive years), our experimental design would be referred as a *repeated measures design* [116,117]. According to repeated measures terms, an individual measured at multiple time points or under different environmental conditions is named the *subject*; factors consisting of levels including independent groups of subjects are named the *between-subjects factor*, whereas time points or environmental conditions are referred to as the repeated factors or the *within-subject factors* [116,117]. Since repeated measurements within subjects are correlated, such data require specific methods for statistical analyses [116,117].

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Two approaches can be used to analyze repeated measures data for one response: analysis of variance with repeated measures (ANOVAR) and multivariate analysis with repeated measures (MANOVAR) [117,118]. In both of these analyses the effects of interest are between-subject effects, within-subject effect, and within-subject-by-between-subjects interactions effects. The test statistics for assessing the significance of these effects can depend on the type of effect. So, for testing the significance of the between-subject effects, the same test can be used in both univariate and multivariate analyses, while for the within subject effects and interactions including these effects, the test statistics are different [117,118].

Profile analysis - To examine whether *in situ* elevated air temperatures can evoke phenotypic responses of functional leaf traits dissimilar than those induced by ambient temperatures, we implemented a multivariate statistical technique known as "profile analysis". Profile analysis is a repeated measures extension of multivariate analysis of variance (MANOVA), which is primarily focused on profiles (vectors) of multivariate data gathered by repeated measurements of a variable from the same individual at several different points in time [117–121]. In this approach, the different measurements conducted on each individual should be considered as multiple dependent variables [117,119]. There are three statistical assumptions underlining profile analysis: (1) the distribution of dependent variables is multivariate normal; (2) the variance-covariance matrix of the variables is homogenous, and (3) the dependent variable are linearly related [117,119,122].

Profile analysis serves to identify whether more than one group of individuals have significantly distinct or similar profiles. Specifically, it quantifies and interprets the amount of variation related to the level and pattern effects. In that context, the mean of a vector of repeated measurements indicates the level of a profile, whereas a vector of differences between each measurement and the average profile level describes the pattern of the profile [119].

Profile plots – To examine the relative behavior of all dependent variables from our data set, we created a profile plot for each leaf trait separately. Profile plots were generated by plotting the sample means of each dependent variable from the two groups within each of the two populations against the measured time points. One of the key objectives for generating a profile plot was to evaluate whether the profiles between the groups are parallel [119].

Profile analysis by groups –The multivariate data for profile analysis were obtained by repeated measurements of a leaf trait on the same clonal individuals from two independent groups that experienced different temperature conditions within their natural habitats. Profiles of the sample means for each group of plants were analyzed as lines in the profile plots. Profile analysis by group implements three tests that examine whether the profiles are parallel, coincidental, and flat between the groups [117,119].

Parallelism is usually the main test in profile analyses because it examines whether each segment of the profile is the same between the groups. A segment is the difference in response between adjacent time points. Parallelism is tested by a one-way MANOVA which compares multiple segments of the profile. This is a test for the *interaction effect* in profile analysis. An interaction happens when the profiles are not parallel. If the null hypothesis of parallelism is rejected, there is a significant interaction between group membership and the time points

Providing that the profiles are parallel, the equal levels hypothesis test whether the profiles of the groups coincide. This test is applied to reveal whether one group on average has higher level than the other over all the time points. To do that, the grand mean of all time points is calculated for each group, and the difference between groups is tested using a univariate test. This is equivalent to the *between-groups main effect* in mixed ANOVA. If the null hypothesis of equal level is rejected, the group levels are significantly different from each other.

Flatness quantifies the degree to which the profiles are level within any group, if parallelism is not rejected. The flatness hypothesis tests whether all dependent variables produce the same mean response. This is equivalent to the *within-subjects main effect* in repeated measures ANOVA. The null hypothesis of flatness is that the grand mean (averaged over treatment levels) of the set of time differences are zero. This is tested by a MANOVA (multivariate *F*). If the flatness hypothesis is rejected, there are differences in the mean values of the variables across multiple time points.

When any of the hypotheses tested by profile analyses are significant then they require to be followed by contrasts.

To identify the particular time interval in which the treatment effects were different, individual ANOVAs (*F*- tests) were implemented on each of the contrasts of interest. We used the Profile transformation to obtain the contrast variables. Profile transformation creates the contrast variables as the differences between adjacent levels of the within-subject data [117]. We tested the significance of both the treatment–by-time interaction as well as the time effect for each contrast of within-subject factor. In these ANOVAs, mean refers to a test for flatness hypothesis, while treatment refers to the test of parallelism hypothesis.

Profile analysis was run using SAS procedure GLM in SAS statistical package, (REPEATED/PROFILE option in the SAS GLM procedure; SAS Institute, 2011) [123].

To determine the strength of statistical association between the pairs of leaf and abiotic variables based on their ranks, Kendall rank correlation analyses was performed (PROC CORR procedure with the KENDALL option in the SAS software; SAS Institute, 2011) [123]. The significance level was set at $p < 0.05$ to determine the statistical significance of the Kendall's Tau correlation coefficients.

To examine the relationship between the air temperature as predictor variable and leaf traits as the outcome variables, robust regression analysis was employed (using PROC ROBUSTREG procedure in the SAS software; SAS Institute, 2011) [123]. The significance level of $p < 0.05$ was chosen to assess the statistical significance of the regression coefficients (b) associated with the predictor variable.

5. Conclusions

Using an open top chamber (OTC) design in this study, each clonal individual of *I. pumila* has simultaneously experienced both the natural and experimentally increased (~1.5 °C) air temperature, enabled us to single out the impact of air temperature from other co-occurring abiotic factors. Our results suggest that phenotypic responses of functional leaf traits to the growth temperature in ramets developed inside and outside of the OTCs were parallel, but not coincidental and flat. This implies that an increase of air temperature for about 1.5° C can evoke changes in both the level and the direction of RNs of functional leaf traits within a single vegetative generation. Since the current amount of plasticity in leaf functional traits of *I. pumila* is molded by natural selection operating in its natural habitats in the past and because the RN shape of these treats expressed at higher air temperature was similar to that recorded at natural temperature, we believe that the amount of plasticity in the four functional leaf traits, SLA, LDMC, SLWC, and LT, is sufficient to shift their phenotypic values toward the fitness optimum in novel environments in the future.

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