Research

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In situ warming in the Antarctic: effects on growth and photosynthesis in Antarctic vascular plants

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Summary

- The Antarctic Peninsula has experienced a rapid warming in the last decades. Although recent climatic evidence supports a new tendency towards stabilization of temperatures, the impacts on the biosphere, and specifically on Antarctic plant species, remain unclear.
- We evaluated the *in situ* warming effects on photosynthesis, including the underlying diffusive, biochemical and anatomical determinants, and the relative growth of two Antarctic vascular species, *Colobanthus quitensis* and *Deschampsia antarctica*, using open top chambers (OTCs) and gas exchange measurements in the field.
- In *C. quitensis*, the photosynthetic response to warming relied on specific adjustments in the anatomical determinants of the leaf CO_2 transfer, which enhanced mesophyll conductance and photosynthetic assimilation, thereby promoting higher leaf carbon gain and plant growth. These changes were accompanied by alterations in the leaf chemical composition. By contrast, *D. antarctica* showed no response to warming, with a lack of significant differences between plants grown inside OTCs and plants grown in the open field.
- Overall, the present results are the first reporting a contrasting effect of *in situ* warming on photosynthesis and its underlying determinants, of the two unique Antarctic vascular plant species, which could have direct consequences on their ecological success under future climate conditions.

Introduction

The Antarctic pearlwort (*Colobanthus quitensis*) and the Antarctic hair grass (*Deschampsia antarctica*) are the only two vascular plant species that have natural populations in the harsh Antarctic environmental conditions (Convey, 1996; Convey & Smith, 2006). Compilation and review studies (e.g. Alberdi *et al.*, 2002; Smith, 2003; Cavieres *et al.*, 2016) have found that there is a combination of morphological and physiological adaptive traits that allows these plant species to withstand the harsh Antarctic climate. For instance, both species deploy xerophytic anatomical characteristics, sufficient freezing tolerance, adequate management of excess photosynthetic active radiation (PAR), and tolerance to water stress (see Cavieres *et al.*, 2016 and references therein). Further, both species are able to grow and maintain substantial photosynthetic rates at low temperatures (e.g. 30% of maximum photosynthesis at 0°C; Xiong *et al.*, 1999), with optimums between 10°C and 19°C (Edwards & Smith, 1988; Xiong *et al.*, 1999; Sierra-Almeida *et al.*, 2007). Although in the field the leaf temperature may episodically reach the optimum temperature for photosynthesis, the leaf temperature remains below that optimum during most of the growing season, suggesting that photosynthesis in the Antarctic vascular plants is often limited by low temperature (Xiong *et al.*, 1999).

The Antarctic Peninsula has experienced rapid warming, exhibiting an average increase in air temperature of *c.* 3.7°C in the latter part of the 20th century (Vaughan *et al.*, 2003; Turner *et al.*, 2013). This warming has entailed longer growing seasons with higher temperatures, ice retreat and higher frequency of rains, which has promoted the increase in cover and number of *D. antarctica* and *C. quitensis* populations (Fowbert & Smith, 1994; Gerighausen *et al.*, 2003; Cannone *et al.*, 2016). Recently,

Turner *et al.* (2016) reported that warming in the Antarctic Peninsula ceased during recent decades, although they warned that new warming episodes are likely to occur in the future. According to Lee *et al.* (2017) this pause in the warming trends is part of the short-term natural climate variability and they anticipate new warming phases across the Antarctic Peninsula during this century.

The response of photosynthesis to changes in temperature reflects a complex interaction between diffusive and biochemical processes (Salvucci & Crafts-Brandner, 2004). Diffusive limitations are associated with the transfer of the atmospheric CO_2 through the boundary layer, stomata and leaf mesophyll to the chloroplast stroma (Evans & Loreto, 2000). Biochemical limitations are related to the capacity of Rubisco to ribulose 1,5-bisphosphate (RuBP) carboxylation, the capacity of electron transfer to RuBP regeneration, and the capacity to regenerate inorganic phosphate for photophosphorylation (Sage *et al.*, 2008). Specific combinations of anatomical traits governing diffusive limitations (Niinemets *et al.*, 2009; Tomás *et al.*, 2013; Flexas *et al.*, 2016) and variation in some Rubisco kinetic parameters affecting the biochemical capacity for CO_2 assimilation (Galmés *et al.*, 2014; Hermida-Carrera *et al.*, 2016; Orr *et al.*, 2016) have been reported in several species under different environmental conditions. In a recent study, we reported that mesophyll conductance (g_m) strongly limits *in situ* carbon assimilation in both Antarctic species (Sáez *et al.*, 2017). The remarkably low g_m values found in these species were found to be related to constitutive leaf anatomical traits (e.g. high cell wall thickness) that are involved in withstanding freezing temperatures, desiccating winds, and low water availability of the Antarctic habitat. Low g_m results in low CO_2 concentration at the carboxylation site, forcing Rubisco from Antarctic vascular plants to specialize in increased specificity for CO_2/O_2, suggesting a compensatory mechanism key for their success in the Antarctic environment (Sáez *et al.*, 2017).

The effects of increased temperature on the performance of the two Antarctic species have primarily been studied in laboratory conditions (Xiong *et al.*, 1999, 2000; Bravo *et al.*, 2007). In an exception, during four growing seasons, Day *et al.* (1999) observed greater above-ground biomass for *C. quitensis* exposed to warmer temperatures, but no significant changes in *D. antarctica*. In addition, Xiong *et al.* (2000) showed that both Antarctic plant species have a low capability for photosynthetic acclimation to temperature in the field. However, there are no studies assessing the *in situ* effect of warming on photosynthesis of the Antarctic vascular plants, their underlying diffusive and biochemical determinants, and their species-specific responses. This information is essential to better forecast the effect of future changes in climate and to determine the mechanisms that Antarctic vascular plants could deploy to cope with predicted climate changes. With this aim, we conducted a climate manipulation experiment using open top chambers (OTCs) in natural populations of *D. antarctica* and *C. quitensis* on King George Island. Based on the role of leaf mesophyll conductance (Sáez *et al.*, 2017), we hypothesize that the photosynthetic response of the Antarctic vascular plants to warmer temperatures relies on specific adjustments in the anatomical determinants of the leaf internal CO_2 transfer, and that photosynthetic acclimation promotes higher rates of net CO_2 assimilation and enhanced growth in these species. However, owing to the lower response capacity previously observed in *D. antarctica* (Day *et al.*, 1999), we suggest that these changes will be more evident in *C. quitensis*.

Materials and Methods

Study site

The study was conducted at the King George Island (Fig. 1) near to the H. Arctowski Polish Antarctic Station (62°09′S, 58°28′W), where both *Deschampsia antarctica* É. Desv. and *Colobanthus quitensis* (Kunth) Bartl. populations are abundant. The selected site for the experiment corresponds to the intermediate vegetation zone after deglaciation described by Kozeretska *et al.* (2010), which is characterized by 100% vegetation cover dominated by bryophytes forming a compact and continuous moss carpet where the two vascular plant species grow interspaced. The most abundant moss species in the carpet are *Sanionia georgico-uncinata*, *Polytrichum piliferum* and *Polytrichastrum alpinum*, and an analysis of the age structure of the *D. antarctica* and *C. quitensis* populations indicated that they were numerically dominated by mature plants (Kozeretska *et al.*, 2010). At this site, *D. antarctica* is more abundant than *C. quitensis*, with densities in the range 70–200 and 20–150 individuals m^{-2}, respectively (Cavieres *et al.*, 2018). Regarding plant sizes at the study site, while *D. antarctica* mats reach a mean size of 10 cm^2, *C. quitensis* individuals reach a mean of 2 cm^2 (Cavieres *et al.*, 2018).

Experimental warming

Ten plots of *c.* 1 m^2 were chosen based on the visual similarity of the vegetation. In each plot, a hexagonal OTC similar to those used in the International Tundra Experiment (ITEX) was installed on December 2012 (Supporting Information Fig. S1). Each OTC was made with transparent Plexiglass® walls of 40 cm height, punched with 25 holes of 1.5 cm diameter each to allow some wind to pass through and hence avoid an excessive increase in the air temperature. These 115 cm basal diameter OTCs are passive warming systems that have been widely used in warming experiments in alpine and arctic tundras (Henry & Molau, 1997; Marion *et al.*, 1997). Additionally, 2 m away (in a random direction) from each installed OTC, a neighboring area with similar characteristics to those where the OTC was placed was selected as a control plot. The spatial arrangement of both OTC and control plots (open areas, hereafter OAs) was random, to avoid any possible effects of OTCs on the neighboring control plots through interference with wind or snow deposition. Although the use of passive warming systems such as OTCs has been controversial (e.g. Kennedy, 1995; De Boeck *et al.*, 2012), some authors argue that OTCs are a reasonable analog of regional warming for remote areas such as polar habitats (Hollister & Webber, 2000; Bokhorst *et al.*, 2013).

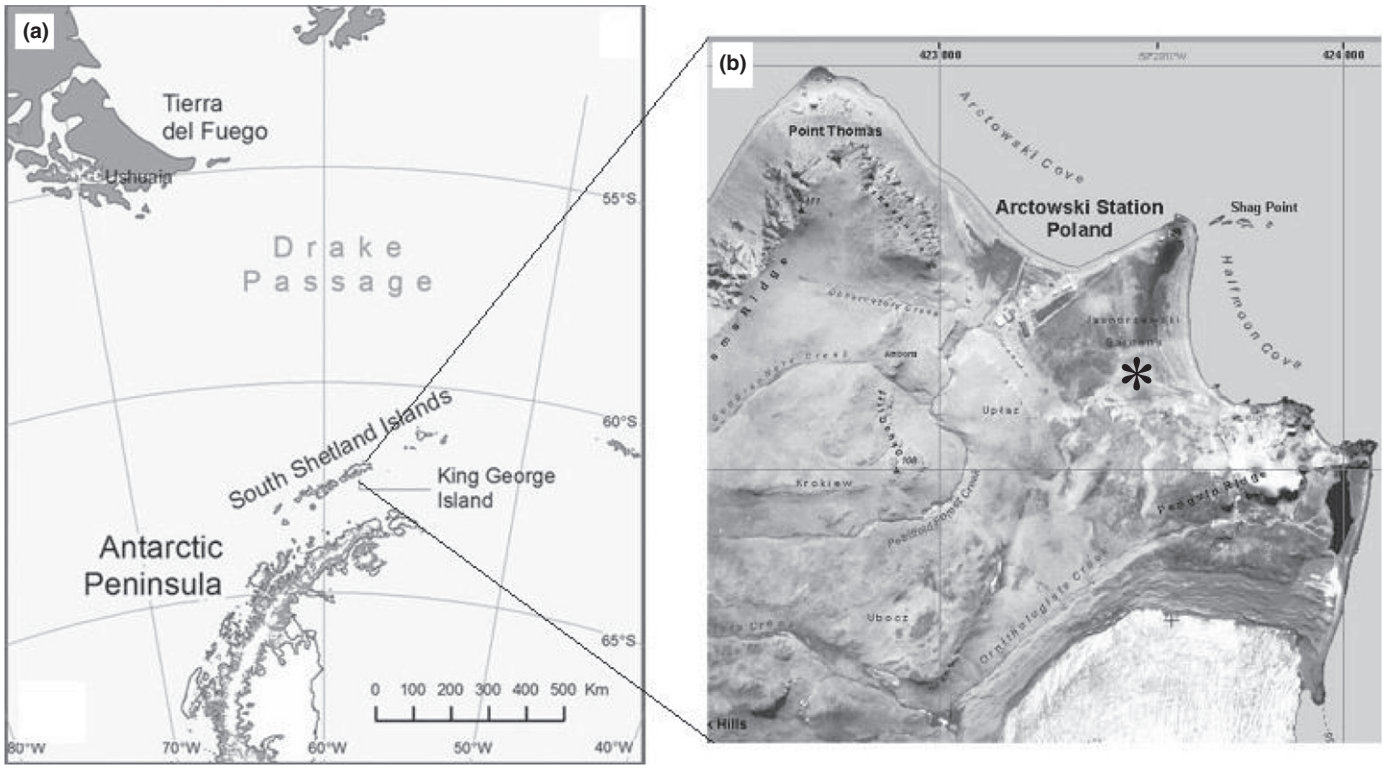


Fig. 1 (a) Study area at King George Island near the Arctowski Polish Antarctic Station (62° 09′ S, 58°28′ W). The asterisk (*) represents the site where open top chambers were installed in December 2012. (b) Close up of area in (a).

For each experimental condition, microclimate measurements were recorded during one growing season (owing to logistic limitations in reaching the study area during each growing season, data were collected from January to March 2015). Temperature and relative humidity (RH) of air at 5 cm above ground level and PAR inside and outside OTC were recorded every hour using two HOBO®-U-30 Stations (Onset Computer Co, Bourne, MA, USA).

Individuals of both species growing in OAs and inside OTCs were randomly selected for growth measurements, leaf photosynthesis and anatomical characterization during February 2015, 3 yr after OTC installation.

Relative growth

The relative growth (RG) of both species was assessed by the increase in basal area of individual mats or rosettes from early December 2014 to early March 2015 (3 months). At the beginning of the growing season (December 2014), 10 individuals of each species, of similar small size, growing inside each OTC were selected, marked and photographed to obtain the initial rosette area of each individual. This same procedure was done in OAs, making a total of 100 individuals per growing condition. At the end of the growing season (March 2015), the same individuals were photographed and these photos were analyzed using image analysis software (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA) to obtain changes in mat or rosette area. Previously, several individuals ($n = 30$) of different sizes were photographed to obtain their mat or rosette areas, and were then collected to obtain their DW. Regression curves between the individual biomass (B_i) as a function of rosette area were determined for both plant species. For *C. quitensis* the best-fit relationship between B_i and rosette areas (A_r) followed a polynomial function ($B_i = 0.0058A_r^2 + 0.0557A_r$; $R^2 = 0.989$, $P < 0.001$), whilst for *D. antarctica*, the best-fit relationship between B_i and A_r followed a linear function ($B_i = 0.0757A_r - 0.2216$; $R^2 = 0.925$, $P < 0.001$). The increase in RG was calculated for each individual as follows: $RG = 100(B_M - B_D)/B_D$, where B_D was plant biomass in December, and B_M was plant biomass in March.

Leaf mass area and leaf density

Leaf mass area (LMA) was calculated as the ratio of dry mass to leaf area and used to convert the area-based parameters into mass-based parameters. For this, six (out of 10) OTCs were randomly selected and one individual of each target species was collected. For OAs, we randomly selected six individuals growing under those conditions. Thus, a total of six replicates were obtained per species and growing condition (OA and OTC). For each collected individual, leaf area was determined for fresh leaves by analyzing the photographs with ImageJ. The dry mass of these leaves was then determined after oven drying for 64 h at 70°C. As LMA only indicates the biomass packaged in one dimension (area), to further analyze how that biomass is packaged along the thickness of the leaf, we estimated the leaf density (LD) by dividing LMA by leaf thickness in both species. The leaf

thickness was obtained from leaf cross-sections analyzed by optical microscopy (see later).

Leaf gas exchange and Chl fluorescence

Leaf gas exchange with Chl*a* fluorescence measurements were recorded using a Li-6400XT, Li-6400-40 leaf chamber (Li-Cor Inc., Lincoln, NE, USA). Six OTCs were randomly selected and one individual of each target species was selected for these measurements. For OAs, we randomly selected six individuals growing under those conditions. For each individual, the gas exchange measurements were done on a group of leaves (from a small branch in *C. quitensis* or a tiller in *D. antarctica*), in an attempt to cover the whole of the infrared gas analyzers's chamber area but avoiding leaf overlap. The actual leaf area in the chamber was estimated and used for measurement corrections. Leaf temperature was measured with the leaf temperature thermocouple (6400-04, Li-Cor Inc.) touching the abaxial surface.

The response of net photosynthesis CO_2 uptake (A_N) to varying substomatal CO_2 concentration (C_i) was determined from A_N–C_i curves in the same way as reported in Sáez *et al.* (2017). Corrections for CO_2 leakage of the leaf chamber of the Li-6400XT were applied to all gas exchange data as described in Flexas *et al.* (2007).

Leaf gas exchange measurements were performed at leaf temperatures of 10°C and 15°C for OAs and OTCs, respectively. Based on the maximal air temperature recorded during the growing season (10 January to 5 March 2015; see the Results section), and the fact that the maximal leaf temperatures are *c.* 4°C above the maximal air temperature during the day (Sáez *et al.*, 2017), we considered these temperatures (10°C and 15°C) to be the maximal leaf temperatures that plants can experience in OAs and OTCs, respectively. It was previously shown that measurement temperatures of 10°C and 15°C did not affect the photosynthetic rate of individual plants of the two species from different provenances within Maritime Antarctica (Sáez *et al.*, 2017).

The quantum efficiency of the photosystem II (PSII)-driven electron transport was determined using the equation $\phi PSII = (F'_m - F_s)/F'_m$, where F_s is the steady-state fluorescence in the light (photosynthetic photon flux density (PPFD) = 1000 μmol quanta $m^{-2}\,s^{-1}$) and F'_m is the maximum fluorescence obtained with a light-saturating pulse (8000 μmol quanta $m^{-2}\,s^{-1}$). As ϕPSII represents the number of electrons transferred per photon absorbed by PSII, the electron transport rate (ETR) can be calculated as $ETR = \phi PSII \times PPFD \times \alpha\beta$, where α is the leaf absorptance and β is the distribution of absorbed energy between the two photosystems, assumed to be 0.5. The leaf absorptance was measured directly in the field as described by Sáez *et al.* (2017) and was 0.729 ± 0.006 with no significant differences between species and growing conditions.

From the combined gas exchange and Chl*a* fluorescence measurement, *in vivo* mesophyll conductance to CO_2 (g_m) was calculated as in Harley *et al.* (1992):

$$g_m = A_N/(C_i - (\Gamma^*(ETR + 8(A_N + R_L))/(ETR - 4(A_N + R_L))))$$

where A_N and C_i were obtained from gas exchange measurements at saturating PPFD (1000 μmol quanta $m^{-2}\,s^{-1}$). The rate of nonphotorespiratory CO_2 evolution in the light (R_L) was assumed to be half of that in the dark (R_{dark}), and the chloroplast CO_2 compensation point (Γ*) was calculated according to Brooks & Farquhar (1985) from the Rubisco specificity factor ($S_{c/o}$) measured *in vitro* (Sáez *et al.*, 2017). The values of g_m were used to calculate the chloroplast CO_2 concentration (C_c), converting $A_N - C_i$ curves into $A_N - C_c$ curves, as $C_c = C_i - (A_N/g_m)$.

The maximum velocity of carboxylation (V_{cmax}) was derived from $A_N - C_c$ curves according to Farquhar *et al.* (1980) and using the *in vitro* Rubisco kinetic constants reported in Sáez *et al.* (2017) for the Antarctic vascular plants. The species-specific values of Γ*, $S_{c/o}$ and the Rubisco Michaelis–Menten constant affinity for CO_2 under 21% O_2 (K_c^{air}) at the different measurement temperatures are provided in Table S1.

Anatomically based modeling of leaf mesophyll conductance

The central portion of leaves of individuals of each species, growing in OAs and inside OTCs (n = 12–18), were collected and fixed in formaldehyde, acetic acid and ethanol and 4% glutaraldehyde, for optical and transmission electron microscopy (JEM1200 EXII; Japan Jeol Ltd, Tokyo, Japan), respectively. Six to ten micrographs were randomly selected to measure leaf anatomical characteristics: mesophyll thickness (T_{mes}); mesophyll area exposed to intercellular air space (S_m) to total leaf surface (S) area ratio (S_m/S); chloroplast exposed surface area to total surface area ratio (S_c/S); distance from the chloroplasts to the cell wall (ΔL_{cyt}); chloroplast length (L_{chl}); chloroplast thickness (T_{chl}) and cell wall thickness (T_{cw}). All images were analyzed using the image analysis software ImageJ.

Leaf anatomical characteristics were used to estimate the modeled mesophyll conductance ($g_{m\ modeled}$) as a composite conductance consisting of within-leaf gas (g_{ias}) and liquid (g_{liq}) components according to the one-dimensional gas diffusion model of Niinemets & Reichstein (2003) as applied by Tomás *et al.* (2013):

$$g_m = 1/((1/g_{ias}) + ((R \cdot T_k)/(H \cdot g_{liq})))$$

where g_{ias} is the gas-phase conductance inside the leaf from substomatal cavities to the outer surface of cell walls, g_{liq} is the conductance in the leaf liquid and lipid phases from the outer surface of cell walls to chloroplasts, R is the gas constant (Pa $m^3\,K^{-1}\,mol^{-1}$), T_k is absolute temperature (K), and H is Henry's law constant for CO_2 (Pa $m^3\,mol^{-1}$). g_m is defined as a gas-phase conductance, and thus $Hl(RT_k)$, the dimensionless form of Henry's law constant, is needed to convert g_{liq} to the corresponding gas-phase equivalent conductance (Niinemets & Reichstein, 2003). The intercellular gas-phase conductance (and the reciprocal, r_{ias}) was obtained according to Niinemets & Reichstein (2003) as:

$$g_{ias} = (1/r_{ias}) = (D_A \cdot f_{ias})/(\Delta L_{ias} \cdot \tau)$$

where ΔL_{ias} is the average gas-phase thickness, τ is the diffusion path tortuosity (Syvertsen *et al.*, 1995), D_A is the diffusivity of CO_2 in air at 10°C and 15°C, and f_{ias} is the fraction of intercellular air spaces. ΔL_{ias} was taken as half the mesophyll thickness. The total liquid phase conductance (g_{liq}) from the outer surface of cell walls to the carboxylation sites in the chloroplasts is the sum of serial resistances of the cell wall (r_{cw}), the plasmalemma (r_{pl}) and inside the cell ($r_{cel,tot}$) (Tomás *et al.*, 2013):

$$g_{liq} = S_m/((r_{cw} + r_{pl} + r_{cel,tot}) \cdot S)$$

The conductance of the cell wall was calculated as previously described by Peguero-Pina *et al.* (2012). For conductance of the plasma membrane, we used an estimate of 0.0035 m s^{-1} as previously suggested (Tosens *et al.*, 2012a). The conductance inside the cell was calculated following the methodology described by Tomás *et al.* (2013), considering two different pathways of CO_2 inside the cell: one for cell wall parts lined with chloroplasts and the other for inter-chloroplastial areas (Tholen *et al.*, 2012).

Measurements of leaf nitrogen, fiber and sclerophylly index

Leaves of individuals of each species, growing in OAs and inside OTCs, were collected in the field and oven-dried for 3 d at 70°C. As the amount of material needed for these analyses was large, we collected material in only three OTCs and three OAs. The leaves were ground with a bead mill (TissueLyser II; Qiagen) and analyzed for fiber content (hemicellulose, cellulose and lignin). Total cell wall fiber, measured as neutral detergent fiber (NDF) and acid detergent lignin, were quantified following the method of Goering & Van Soest (1970). Total leaf nitrogen concentration was measured using an Organic Elemental Analyzer (Flash EA 112; Thermo Fisher Scientific Inc., Waltham, MA, USA). The Loveless sclerophylly index was calculated according to Read *et al.* (2016) as NDF per unit protein ($N_{mass} \times 6.25$).

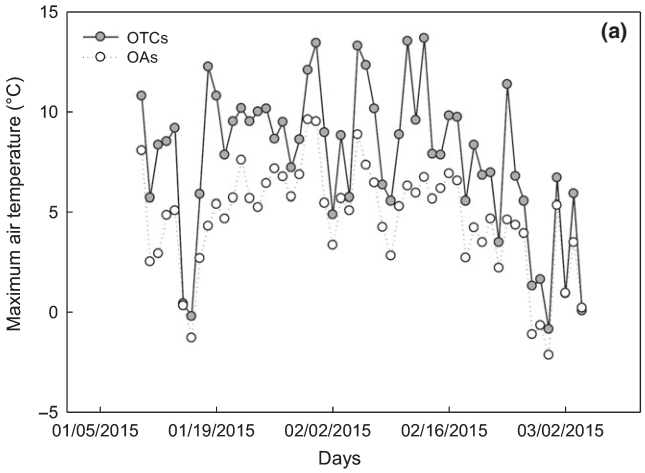
Statistical analyses

The effects of growing condition (OA and OTC) on the relative growth, leaf anatomy, leaf chemistry and photosynthetic performance were assessed for each plant species with Student's *t*-test ($\alpha = 0.05$). Analyses were performed with the SPSS statistics 19.0 software package (IBM software, New York, NY, USA). A correlation analysis was performed to assess the relationships between A_N and g_m and V_{cmax}, and between $g_{m\ modeled}$ and different anatomical traits. These analyses were done with STATISTICA 7.0 (Stat Soft Inc. Tulsa, OK, USA).

Results

Microclimatic conditions and relative growth

Air temperatures during the day between 10 January and 5 March 2015 were significantly affected by OTCs (Fig. 2).



(b)

	Mean air temp. (°C)	Mean Max Air temp. (°C)	Mean Min Air temp. (°C)	Time above 0°C (h)	Time 5–10°C (h)	Time above 10 °C (h)
OAs	2.71 ± 0.31	4.83 ± 0.40	0.71 ± 0.30	1066	163	7
OTCs	4.08 ± 0.34*	7.81 ± 0.50*	0.92 ± 0.30	1071	317	31

Fig. 2 (a) Daily maximum air temperature at the studied site, recorded in open areas (OAs) and inside open top chambers (OTCs) between 10 January and 5 March 2015. Data of average, maximum and minimum air temperatures, and the number of hours with temperatures above 0°C, in the ranges of 5–10°C and > 10°C in OAs and OTCs (b). Values are means ± SEM. Significant differences between OAs and OTCs according to Student's *t*-test: *, $P < 0.05$. Date format in (a) is month/day/year.

Although minimum mean air temperatures were very similar between OAs and OTCs (0.7 vs 0.9°C, respectively), the overall mean air temperature was significantly higher inside OTCs (2.7°C and 4.1°C for OAs and OTCs, respectively). The maximum mean air temperatures was *c.* 3°C higher inside OTCs (4.8 ± 0.4°C in OAs and 7.8 ± 0.5°C inside OTCs). As a proxy of the accumulated amount of heat during the growing season inside and outside OTCs, we determined the number of hours with air temperatures between 5°C and 10°C, and above 10°C. OA plants experienced 163 h with air temperatures between 5°C and 10°C, whereas this value was twofold higher inside OTCs (317 h). Likewise, air temperatures were above 10°C for 7 h in OAs, whereas this value reached 31 h in OTCs. Other parameters such as mean PAR (400 ± 38 and 400 ± 51 µmol photon m^{-2} s^{-1} in OAs and OTCs, respectively) and RH during the day were not altered by the OTCs (Fig. 2b).

Regarding growth and most of the physiological parameters evaluated, *D. antarctica* and *C. quitensis* responded differently to the *in situ* warming. Whereas in *D. antarctica* the RG was not affected by warming, showing an increase of *c.* 28% from December 2014 to March 2015 for both OA and OTC plants (Fig. 3a), the RG of *C. quitensis* was positively affected by warming, leading to a greater increase in biomass, from 42.3 ± 4.8% in OAs to 73.6 ± 8.7% in OTCs (Fig. 3b). In the same way, *D. antarctica* LMA and LD values did not change with the growing condition, with LMA values *c.* 0.2 kg m^{-2} and LD values *c.* 1.5 g cm^{-3} (Fig. 3c,e). By contrast, *C. quitensis* LMA differed

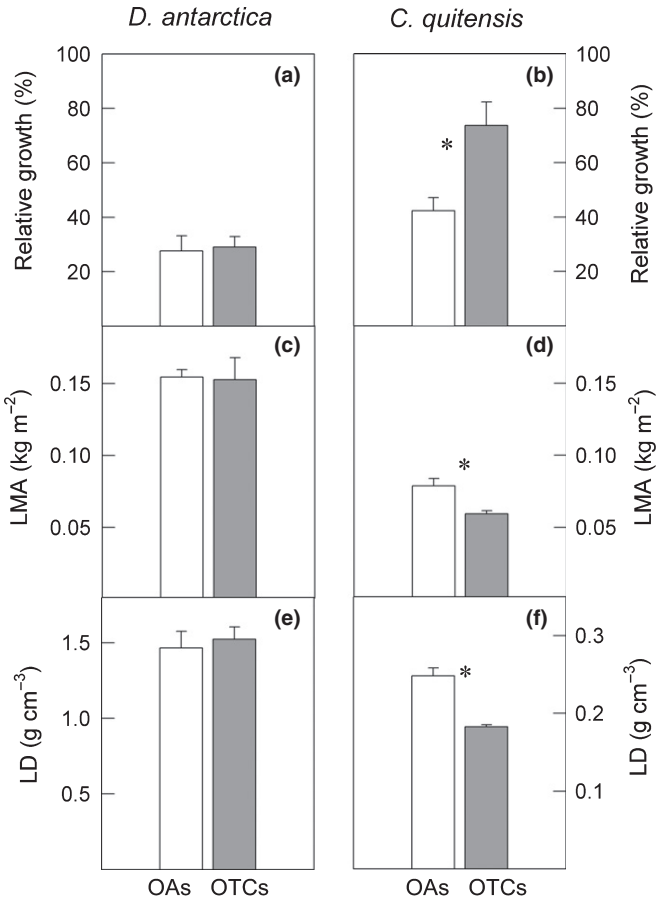


Fig. 3 The relative growth (a, b), the leaf mass area (LMA; c, d) and the leaf density (LD; e, f) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (OAs) and inside open top chambers (OTCs). Values are means ± SEM ($n = 100$ for relative growth and $n = 6$ for LMA and LD). Significant differences between OAs and OTCs for each species according to Student's *t*-test: *, $P < 0.05$.

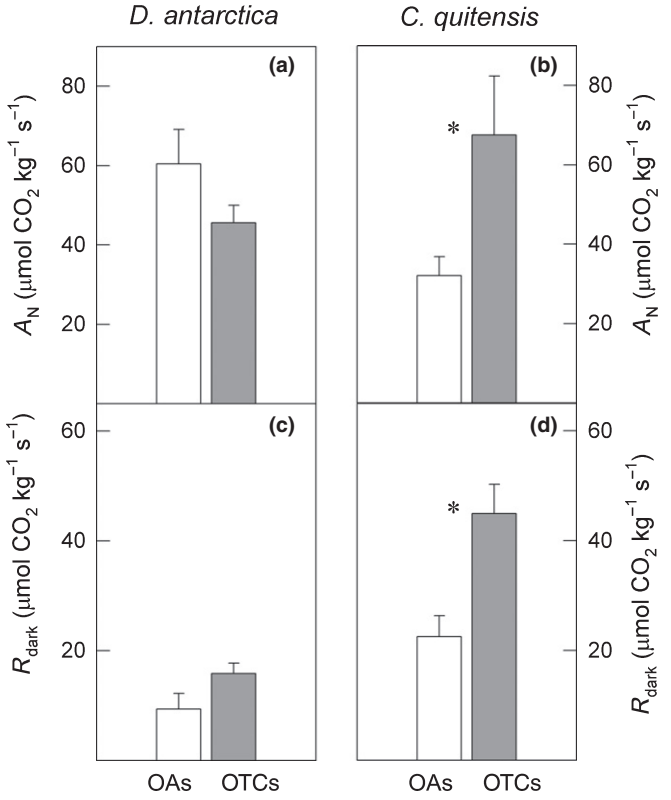


Fig. 4 The net photosynthetic CO_2 assimilation rate (A_N; a, b) and the dark respiration (R_{dark}; c, d) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (OAs) and inside open top chambers (OTCs). Values are means ± SEM ($n = 6$). Significant differences between OAs and OTCs for each species according to Student's *t*-test: *, $P < 0.05$.

between OA plants (0.08 ± 0.00 kg m^{-2}) and those growing inside OTCs (0.06 ± 0.00 kg m^{-2}; Fig. 3d), with the same trend observed for LD (0.25 ± 0.01 g cm^{-3} for OA plants vs 0.18 ± 0.003 g cm^{-3} for OTC plants; Fig. 3f).

The response of photosynthesis and its underlying determinants to field warming conditions

In both species, no differences between OAs and OTCs were detected for photosynthesis and its determinants when expressed on an area basis. However, in line with the LMA and LD results, when photosynthetic parameters were expressed on a mass basis, most differed between growing conditions for *C. quitensis*, but not for *D. antarctica* (Fig. 4). In this latter species, the mass-based net photosynthesis at ambient CO_2 concentration (A_N) and the mitochondrial dark respiration rate (R_{dark}) were *c.* 53.0 and 12.6 μmol CO_2 kg^{-1} s^{-1}, respectively (Fig. 4a,c). Conversely, in *C. quitensis*, mass-based A_N was twofold higher in OTC plants (67.5 ± 14.9 μmol CO_2 kg^{-1} s^{-1}) than in OA plants (32.1 ± 4.7 μmol CO_2 kg^{-1} s^{-1}) (Fig. 4b). Likewise, mass-based R_{dark} was also higher in OTC plants (44.9 ± 5.3 μmol CO_2 kg^{-1} s^{-1}) than in OA plants (22.5 ± 3.8 μmol CO_2 kg^{-1} s^{-1}) (Fig. 4d). The contrasting response of the CO_2 assimilation capacity between the two Antarctic species to *in situ* warming was, in most cases, related to contrasting changes in the diffusive and biochemical determinants (Table 1).

Although in both species *in situ* warming tended to reduce diffusive limitations associated with stomatal conductance (g_s), this reduction was significant only in *D. antarctica*. For this species, the *in vivo* g_m was similar between OA and OTC plants, while chloroplast CO_2 concentration (C_c) and maximum Rubisco carboxylation rate (V_{cmax}) also showed no differences between OA and OTC plants (Table 1). By contrast, in *C. quitensis* g_m was significantly higher in OTC plants than in OA plants (0.2 ± 0.0 mol CO_2 kg^{-1} s^{-1} and 0.1 ± 0.0 mol CO_2 kg^{-1} s^{-1}, respectively). *In situ* warming also resulted in a higher C_c in OTC plants (77.3 ± 10.6 μmol CO_2 mol air^{-1}) than in OA plants (47.5 ± 3.1 μmol CO_2 mol air^{-1}) and higher V_{cmax} in OTC plants (581.4 ± 36.3 μmol CO_2 kg^{-1} s^{-1}) compared with OA plants (336.2 ± 31.9 μmol CO_2 kg^{-1} s^{-1}; Table 1). Despite the different responses found between the two Antarctic species, in both species g_m was significantly correlated with A_N ($P < 0.001$, Fig. 5a,b) and a significant correlation between V_{cmax} and A_N was only found in *C. quitensis* (Fig. 5c,d).

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Table 1 The stomatal conductance (g_s), *in vivo* leaf mesophyll obtained with Harley's method (g_m), chloroplast CO_2 concentration (C_c) and maximum Rubisco carboxylation rate (V_{cmax}) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (OAs) and inside warming chambers (OTCs)

	Deschampsia antarctica		*Colobanthus quitensis*	
Parameter	OAs	OTCs	OAs	OTCs
g_s (mol H_2O m^{-2} s^{-1})	0.15 ± 0.02	0.10 ± 0.01*	0.15 ± 0.02	0.09 ± 0.02
g_m (mol CO_2 kg^{-1} s^{-1})	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*
C_c (μmol CO_2 mol air^{-1})	81.8 ± 11.7	104.3 ± 18.3	47.5 ± 3.1	77.3 ± 10.6*
V_{cmax} (μmol CO_2 kg^{-1} s^{-1})	315.5 ± 12.7	278.0 ± 30.6	336.2 ± 31.9	581.4 ± 036.3*

Values are means ± SEM ($n = 6$). Significant differences between OAs and OTCs in each species according to Student's *t*-test: *, $P < 0.05$.

Anatomical traits governing the mesophyll conductance to CO_2

The g_m values estimated *in vivo* by Harley's method were confirmed by the anatomical measurements of mesophyll conductance ($g_{m\ modeled}$). Regardless of the growing conditions, *D. antarctica* showed no changes in $g_{m\ modeled}$ in most of the ultrastructural components associated with this trait, except for those associated with chloroplast size (Table 2). Inside the OTCs, the average distance between chloroplasts and the cell wall (ΔL_{cyt}) was greater than in the OAs (ranging from 0.154 ± 0.05 μm in OA plants to 0.38 ± 0.088 μm in OTC plants). In addition, chloroplasts were smaller in OTC than in OA plants. By contrast, *C. quitensis* deployed a higher $g_{m\ modeled}$ in OTC plants, and several adjustments in other anatomical traits were also observed (Table 2). Although for this species warming produced no significant variation in mesophyll thickness (T_{mes}) and cell wall thickness (T_{cw}), there were differences in ΔL_{cyt} and chloroplast thickness (T_{chl}). In OTC plants, ΔL_{cyt} (0.217 ± 0.03 μm) and T_{chl} (2.22 ± 0.20 μm) were lower than in OA plants (0.535 ± 0.06 and 5.12 ± 0.67 μm, respectively).

Likewise, *C. quitensis* growing inside OTCs presented higher mesophyll surface area facing intercellular air spaces (S_m/S), being almost twofold higher in OTC plants than in OA plants (9.25 ± 0.52 and 4.34 ± 0.48 m^2 m^{-2}, respectively). For this species, differences in the anatomical component for CO_2 diffusion were also observed. Specifically, the conductance of the liquid phase (g_{liq}) was higher in OTC plants, so that mesophyll conductance to CO_2 diffusion was higher in *C. quitensis* growing under the *in situ* warming condition (Table 2). In addition, in *C. quitensis*, strong correlations were found between $g_{m\ modeled}$ and LMA ($R = -0.766$, $P < 0.001$), LD ($R = -0.692$, $P < 0.05$) and S_m/S ($R = 0.821$, $P < 0.001$). These relationships were significant for *D. antarctica* only between $g_{m\ modeled}$ and S_m/S ($R = 0.704$, $P < 0.05$; Fig. 6).

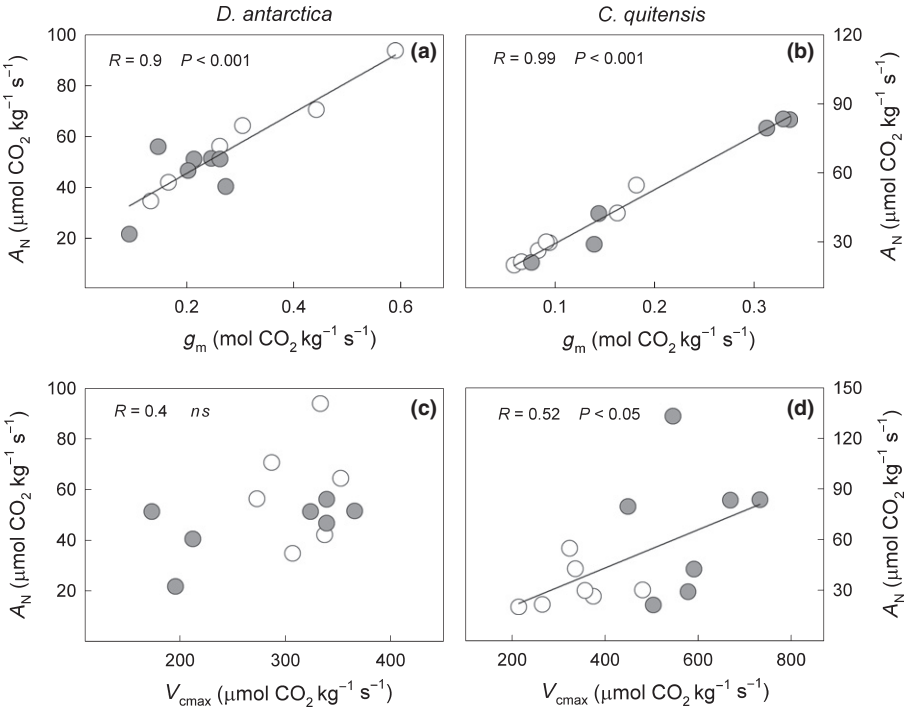

Fig. 5 The relationship between the dry mass-based net CO_2 assimilation rate (A_N) and the leaf mesophyll CO_2 conductance (g_m; a, b) and the maximum Rubisco carboxylation rate (V_{cmax}; c, d) in *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (open circles) and inside open top chambers (closed circles). Regression coefficient and the significance of the relationship are shown for each species considering both growing conditions together. ns, not significant.

Table 2 The leaf mesophyll conductance modeled with anatomical parameters (g_m), the mesophyll thickness (T_{mes}), average distance between the chloroplasts and the cell wall (ΔL_{cyt}), chloroplast thickness (T_{chl}), chloroplast length (L_{chl}), cell wall thickness (T_{cw}), mesophyll (S_m/S) and chloroplast (S_c/S) surface area facing intercellular air spaces per leaf area, intercellular air space (g_{ias}) and liquid phase (g_{liq}) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (OAs) and inside warming chambers (OTCs)

	Deschampsia antarctica		*Colobanthus quitensis*	
Parameter	OAs	OTCs	OAs	OTCs
g_m (mol CO_2 kg^{-1} s^{-1})	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1*
T_{mes} (μm)	110.14 ± 5.78	99.89 ± 3.99	322.30 ± 13.40	325.80 ± 5.00
T_{cw} (μm)	0.25 ± 0.01	0.27 ± 0.02	0.35 ± 0.06	0.33 ± 0.02
ΔL_{cyt} (μm)	0.15 ± 0.05	0.38 ± 0.09*	0.54 ± 0.06	0.22 ± 0.03*
T_{chl} (μm)	3.57 ± 0.25	2.91 ± 0.11*	5.12 ± 0.66	2.22 ± 0.20*
L_{chl} (μm)	5.55 ± 0.26	4.83 ± 0.21*	5.33 ± 0.29	5.19 ± 0.35
S_m/S (m^2 m^{-2})	6.24 ± 0.58	6.49 ± 0.51	4.34 ± 0.48	9.25 ± 0.52*
S_c/S (m^2 m^{-2})	1.81 ± 0.33	2.24 ± 0.48	2.06 ± 0.32	2.23 ± 0.32
g_{ias} (m s^{-1})	0.043 ± 0.005	0.044 ± 0.003	0.013 ± 0.002	0.014 ± 0.001
g_{liq} (m s^{-1})	0.0005 ± 0.000	0.0005 ± 0.000	0.0003 ± 0.000	0.001 ± 0.000*

Values are means ± SEM (n = 6–10). Significant differences between OAs and OTCs in each species according to Student's t-test: *, $P < 0.05$.

The effect of warming on leaf nitrogen, fiber and sclerophylly index

In *D. antarctica,* a notably higher total fiber content was found in OTC plants (40.52 ± 0.30%) than in OA plants (38.68 ± 0.44%), which was mainly a result of a higher cellulose content determined in OTC (Table 3). Hemicellulose and lignin content were not affected by *in situ* warming. In addition, the area-based leaf nitrogen content was lower in OTC plants and, therefore, the Loveless sclerophylly index (SI) was higher. Conversely, in *C. quitensis* total fiber content was significantly lower in OTC plants (25.24 ± 0.38%) than in OA plants (29.69 ± 0.15%), whereas there was significantly lower hemicellulose, cellulose and lignin content in OTC plants. Nitrogen content was lower in OTC plants, and no differences were detected in SI (Table 3).

Discussion

In situ warming and its differential effect on RG and photosynthesis of two Antarctic vascular species

The forecasts for the Antarctic Peninsula project are for increases in summer air temperature of *c.* 3°C by 2030 (Mitchell *et al.*, 1990). These estimates fit well with the increased temperature in our experimental setup (Fig. 2), which constitutes a good warming simulation. However, other physical environmental variables not studied (such as snow prevalence and wind reduction) should be considered in future experiments. While in this work leaf temperature data were not shown, we previously showed that in the same field conditions, the maximum leaf temperature was *c.* 4°C higher than the air temperature during the day (Sáez *et al.*, 2017). These data confirm previous reports indicating that the canopy temperature under Antarctic conditions can be considerably higher than ambient air temperature (Smith, 2003; Casanova-Katny *et al.*, 2010). Overall, these observations and the average air temperatures recorded in the present study (Fig. 2) confirm that plants inside OTCs grew at higher temperatures during three growing seasons.

It has been suggested that warmer air temperatures, such as those generated inside the OTCs, should promote the growth of the two Antarctic vascular species as they are exposed to temperatures closer to the optimal for photosynthesis (Xiong *et al.*, 1999). Indeed, expansions in both population sizes and localities have been reported for the two species along the Antarctic Peninsula and adjacent islands and they have been regarded as a clear consequence of the climate warming observed in the area (Fowbert & Smith, 1994; Gerighausen *et al.*, 2003; Torres-Mellado *et al.*, 2011). Cannone *et al.* (2016), in a detailed comparison of the cover and number of localities of both Antarctic plant species in the Argentine Islands between 1960 and 2009, showed that *D. antarctica* increased its cover and number of localities by 191% and 104%, respectively. By contrast, *C. quitensis* showed increases of 208% and 35% in its cover and number of localities, respectively. Thus, the two species responded quite differently to the increase in temperature observed during that period; while warming promoted *D. antarctica* colonization rather than its growth, and hence cover, the opposite was observed in *C. quitensis* (Cannone *et al.*, 2016). As previously indicated, and in support of the present findings, Day *et al.* (1999) observed a contrasting response to experimental warming among the two Antarctic species, similar to what is reported here. According to our results, this differential response to higher temperatures relied on specific adjustments in the leaf anatomical determinants of CO_2 transfer that only occurred in *C. quitensis*, which promoted higher rates of net CO_2 assimilation and enhanced growth under warming conditions (these results are summarized in Fig. 7). By contrast, *D. antarctica* showed no increased growth under warming, and also no changes in carbon gain (i.e. no changes in A_N and R_{dark}), and no changes in their anatomical traits (Fig. 3). Our findings agree with the results obtained by Xiong *et al.* (2000), where *D. antarctica* plants exposed to increases in temperature under laboratory conditions showed no changes in photosynthesis and plants had a similar optimal temperature for

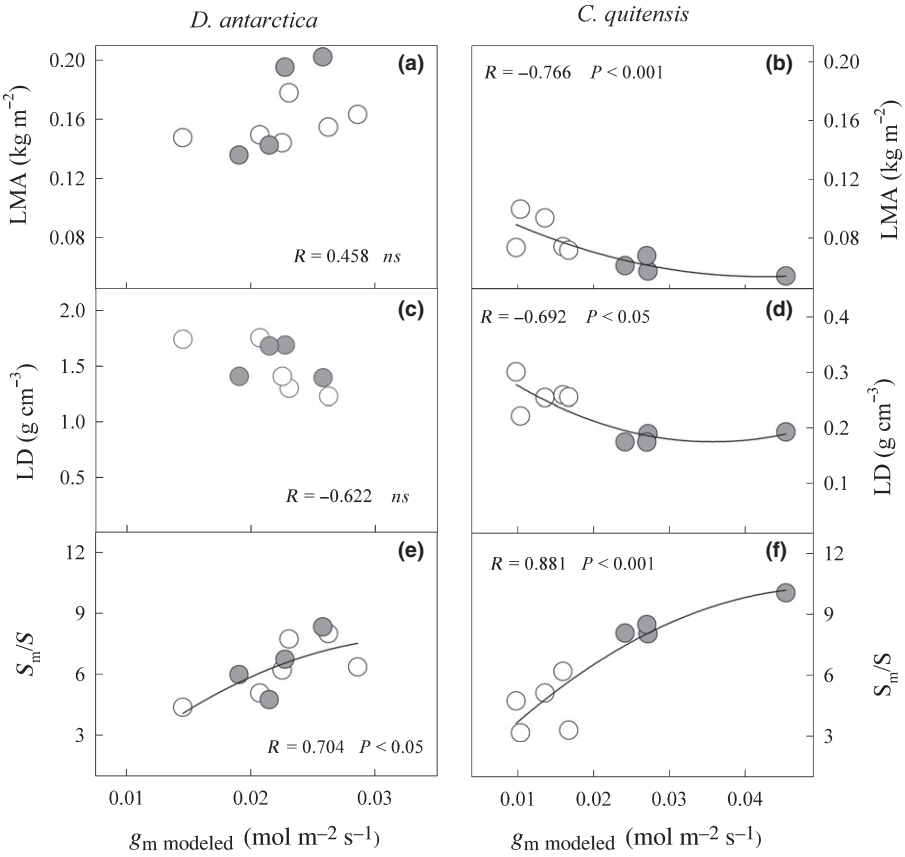

Fig. 6 Correlations of the mesophyll leaf conductance modeled with anatomical parameters ($g_{m\ modeled}$) with the leaf mass area (LMA; a, b), the leaf density (LD; c, d) and the mesophyll surface area facing intercellular air spaces per leaf area (S_m/S; e, f) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (open circles) and inside open top chambers (OTC; closed circles). Regression coefficient and the significance of the relationship are shown for each species considering both growing conditions together.

Table 3 The fiber concentration (hemicellulose, cellulose and lignin) for *Deschampsia antarctica* and *Colobanthus quitensis* growing in open areas (OAs) and inside warming chambers (OTCs)

	Deschampsia antarctica		*Colobanthus quitensis*	
Parameter	OAs	OTCs	OAs	OTCs
Total fiber (% DW)	38.68 ± 0.44	40.52 ± 0.30*	29.69 ± 0.15	25.24 ± 0.38*
Hemicellulose (% DW)	24.44 ± 0.36	25.30 ± 0.23	18.63 ± 0.16	16.02 ± 0.28*
Cellulose (% DW)	12.63 ± 0.05	13.70 ± 0.15*	10.85 ± 0.01	9.13 ± 0.10*
Lignin (% DW)	1.61 ± 0.04	1.53 ± 0.02	0.21 ± 0.01	0.09 ± 0.01*
Nitrogen (%DW)	1.63 ± 0.06	1.31 ± 0.07*	1.80 ± 0.09	1.53 ± 0.06*
Loveless sclerophylly index	3.85 ± 0.14	5.01 ± 0.27*	2.67 ± 0.13	2.66 ± 0.10

Values are means ± SEM ($n = 3$). Significant differences between OAs and OTCs in each species according to Student's *t*-test: *, $P < 0.05$.

photosynthesis (10°C). Similarly, Edwards & Smith (1988) grew plants under UK summer conditions and showed that photosynthesis and respiration of *D. antarctica* did not differ from plants that never experienced those warmer conditions. These authors concluded that in this species, enzymatic properties and metabolism must be under strict genetic control, in contrast to the greater phenotypical and functional plasticity of *C. quitensis*. The capacity of *C. quitensis* to carry out photosynthesis over a wider temperature range than *D. antarctica* supports this greater metabolic flexibility, which is consistent with the higher optimal temperature determined in *C. quitensis* compared with *D. antarctica* (Edwards & Smith, 1988; Xiong *et al.*, 1999).

A key leaf trait that determines the rate of photosynthesis is mesophyll conductance to CO_2 diffusion (Niinemets *et al.*, 2009; Peguero-Pina *et al.*, 2015). Recently, we reported g_m in *D. antarctica* and *C. quitensis* from distant Antarctic populations and found that their g_m values are among the lowest for higher plants so far (Sáez *et al.*, 2017). In addition, in that study, the g_s was relatively high compared with g_m, which would help to offset some of the diffusive limitation. In the present study, as with the results for RG and A_N, only *C. quitensis* increased g_m under warmer conditions, reaching values about threefold higher in OTC plants than in OA plants (Table 1). In line with this, a strong positive correlation was found between mass-based A_N vs g_m (Fig. 5), in accordance with previous reports for Antarctic plants (Sáez *et al.*, 2017) and other species (Evans & Loreto, 2000; Niinemets & Sack, 2006; Warren, 2008). The absence of correlation between A_N and V_{cmax} in *D. antarctica* and the lower

significance of this correlation in *C. quitensis* confirm that the increase in CO_2 availability is affecting A_N to a greater extent than the enzymatic Rubisco properties at saturating CO_2 concentrations. Thus, the higher mass-based g_m determined in *C. quitensis* inside OTCs resulted in almost twofold chloroplast CO_2 concentration (C_c, Table 1), significantly reducing the diffusional limitations and, hence, enhancing the rate of CO_2 assimilation (Fig. 1b). In addition, the higher V_{cmax} in this species was accompanied by a lower LMA and LD (Fig. 4). Thus, as previously reported by Boese & Huner (1990), in *C. quitensis*, warmer temperatures seem to be associated with greater leaf expansion and lower leaf density, perhaps to maximize the efficiency of g_m as the most limiting factor for photosynthesis. It has been proposed that in species with higher LMA, photosynthesis is more limited by g_m (reviewed by Flexas *et al.*, 2008; Niinemets *et al.*, 2009). However, *D. antarctica*, with an LMA higher than that of *C. quitensis*, displayed a higher *in vivo* g_m regardless of growth conditions. This may be a result of anatomical and/or biochemical adaptations that may offset the restrictions imposed by high LMA in g_m and A_N (Peguero-Pina *et al.*, 2017a). The high LMA shown by *D. antarctica* constitutes an important trait that allows this species to flourish in the xeric Antarctic environment.

Changes in diffusive limitations with warming are driven by leaf anatomical and chemical modifications

The specific modification exerted by OTCs on the $g_{m\ modeled}$ through anatomical determinants and the leaf chemical composition resemble the trends of *in vivo* g_m estimated with Harley's method (Tables 2, 3). In *D. antarctica*, $g_{m\ modeled}$ and their underlying anatomical traits were, for the most part, not altered by OTC, except for those associated with chloroplast size. In general, OTC plants showed smaller chloroplasts and these were further away from the cell wall (ΔL_{cyt}). In this species, as in several polar and alpine plants (Lütz *et al.*, 2012), chloroplast size has been indicated as a flexible character that responds to environmental factors such as temperature (Jellings *et al.*, 1983; Giełwanowska *et al.*, 2015). However, these changes do not seem to trigger a direct effect on g_m.

On the other hand, for *C. quitensis*, $g_{m\ modeled}$, like *in vivo* g_m, was higher in plants growing under *in situ* warming. As in this species g_m is notably low and negatively correlated with LMA and LD (Fig. 6). Such changes are reflective of a structural control on the mesophyll diffusion limitations of photosynthesis. This, together with the greater proximity of chloroplasts to the cell wall, lower T_{chl}, and higher S_m/S (Table 2; Fig. 7), constitutes an important anatomical factor that favors an enhancement of leaf internal CO_2 transfer under warming conditions (Peguero-Pina *et al.*, 2012, 2015; Tosens *et al.*, 2012b; Adachi *et al.*, 2013). Indeed, a strong correlation between $g_{m\ modeled}$ and S_m/S has been observed worldwide (Wright *et al.*, 2004; Peguero-Pina *et al.*, 2015) and also in *C. quitensis* (Fig. 6f). Consistent with this, the lower LD observed in *C. quitensis* OTC plants (Fig. 3f) is also associated with a higher gas-phase volume (for reviews see Niinemets, 1999; Niinemets & Sack, 2006), and this increased liquid-phase diffusion conductance (Table 2), and therefore g_m (Terashima *et al.*, 2005; Evans *et al.*, 2009).

In addition, *C. quitensis* plants grown in OTCs exhibited a lower fraction of cell wall chemical components (hemicellulose, cellulose and lignin content) and leaf nitrogen content (Table 3), allowing a better CO_2 transference into the leaf mesophyll (Wright & Cannon, 2001; Wright *et al.*, 2004; Flexas *et al.*, 2014). Although increases in leaf nitrogen content have been associated with a greater V_{cmax} (Peguero-Pina *et al.*, 2017b), the higher nitrogen content in *C. quitensis* seems to be associated with an increase in foliage robustness to the harsh conditions in OAs, as previously reported by Niinemets (2015) for other species.

All in all, we contend that the plasticity of *C. quitensis* may result in higher photosynthesis rates in response to warmer conditions. This plasticity may be, at least partially, a consequence of the observed coordination between morpho-

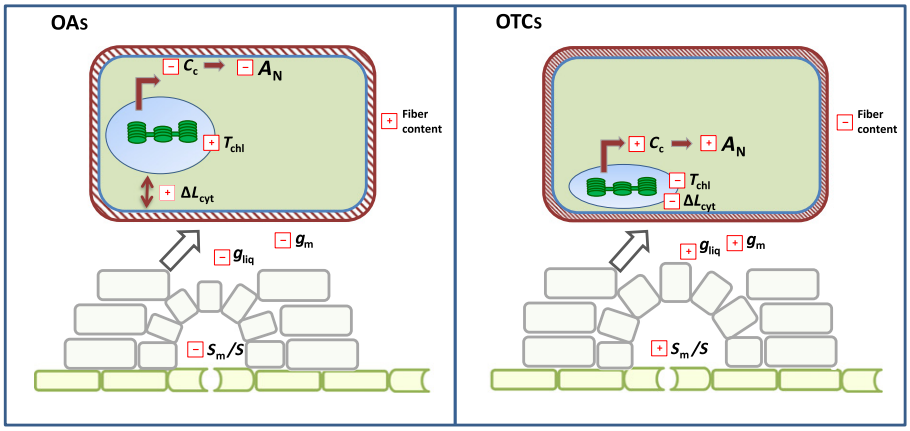

Fig. 7 Conceptual scheme showing the anatomical and biochemical traits in response to warming (open top chambers (OTCs)) and their consequences for the CO_2 assimilation observed in *Colobanthus quitensis*, but not in *Deschampsia antarctica*. S_m/S, chloroplast-exposed surface area to total surface area ratio; g_{liq}, liquid phase; g_m, leaf mesophyll conductance to CO_2; ΔL_{cyt}, distance from the chloroplast to the cell wall; T_{ch}, chloroplast thickness; C_c, chloroplast CO_2 concentration; A_N, net photosynthesis. Plus and minus signs in the blocks indicate the lower or higher value of the respective parameter. OAs, open areas.

anatomical and chemical traits, which in turn results in maximizing total leaf conductance to CO_2, biochemical performance, carbon gain and relative growth. Conversely, in *D. antarctica*, warmer conditions lead to higher fiber content, mainly cellulose and higher sclerophylly (Table 3). These results are consistent with those reported by Romero *et al.* (1999) and Gielwanowska *et al.* (2005), who found a higher number of sclerenchymatic fibers in leaves of plants in a growth chamber, compared with those in the field. It seems that the stressful Antarctic field conditions stimulate synthesis of compounds that slow down elongation growth, altering the cell wall lignification (Gielwanowska *et al.*, 2005). Nonetheless, these chemical changes had no effect on the mesophyll CO_2 transfer. Thus, the small effect of warming on *D. antarctica* suggests that this species seems not to be limited by low temperature.

Given that plant responses to environmental manipulations in other cold climates, such as the Arctic, have been shown to take several years to manifest (Hollister *et al.*, 2005; Elmendorf *et al.*, 2012), this raises the question of whether the differential response in *C. quitensis* and *D. antarctica* is just a matter of timescale. In other words, would *D. antarctica* show a similar response in a longer-term observation period? On top of this, we consider that long-term field studies are needed to make accurate predictions regarding the biological consequences of global warming on Antarctic plants.

Concluding remarks

The present study established a causal link among anatomical and chemical adjustment, mesophyll conductance, CO_2 assimilation, and therefore RG under *in situ* warming conditions, in which the Antarctic species *C. quitensis* showed changes that resulted in greater growth, while *D. antarctica* showed no changes in its anatomical traits and no changes in growth (to summarize the response shown see Fig. 7). *C. quitensis* seems to be limited by the low Antarctic summer temperature, and warming brings it closer to its optimal temperature for growth. Thus, the increased RG in *C. quitensis* is produced by an improvement in leaf carbon gain, where A_N is comparatively more favored by temperature than R_{dark}. The higher A_N under warming is related to a higher g_m, which can be explained by the occurrence of higher S_m/S and lower LMA, LD, ΔL_{cyt}, T_{chl} and fiber content (plus and minus signs in Fig. 7). By contrast, *D. antarctica* was less responsive, showing no changes to warmer conditions, at least in the studied parameters. Given that plant responses to environmental manipulations in other cold climates, such as the Arctic, can take several years to manifest, it seems likely that changes in *D. antarctica* could be detected after a much longer period under environmental warming.

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Author contributions

P.L.S., L.A.C., J.G., L.J.C. and L.A.B. planned and designed the research.

P.L.S., L.A.C., L.A.B., C.S., C.F.R., B.K.R. and M.V. performed the field measurements. E.G-P., J.J.P-P. and D.S-K. performed the chemical analysis. P.L.S., L.A.C., L.A.B., J.G., C.F.R., B.K.R. and J.J.P-P. analyzed the data. P.L.S. wrote most of the manuscript but with substantial contributions from L.A.C., J.G., L.A.B., E.G-P. and J.J.P-P.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Images of the OTCs *in situ* at King George Island.

Table S1 The chloroplastic CO_2 compensation point (Γ*), Rubisco specificity factor ($S_{c/o}$) and Rubisco Michaelis–Menten constant affinity for CO_2 (K_c^{air}) for the two Antarctic species

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