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Symbiosis at its limits: ecophysiological consequences of lichenization in the genus *Prasiola* in Antarctica

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• **Background and Aims** Lichens represent a symbiotic relationship between at least one fungal and one photosynthetic partner. The association between the lichen-forming fungus *Mastodia tessellata* (Verrucariaceae) and different species of *Prasiola* (Trebouxiophyceae) has an amphipolar distribution and represents a unique case study for the understanding of lichen symbiosis because of the macroalgal nature of the photobiont, the flexibility of the symbiotic interaction and the co-existence of free-living and lichenized forms in the same microenvironment. In this context, we aimed to (1) characterize the photosynthetic performance of co-occurring populations of free-living and lichenized *Prasiola* and (2) assess the effect of the symbiosis on water relations in *Prasiola*, including its tolerance of desiccation and its survival and performance under sub-zero temperatures.
• **Methods** Photochemical responses to irradiance, desiccation and freezing temperature and pressure–volume curves of co-existing free-living and lichenized *Prasiola* thalli were measured *in situ* in Livingston Island (Maritime Antarctica). Analyses of photosynthetic pigment, glass transition and ice nucleation temperatures, surface hydrophobicity extent and molecular analyses were conducted in the laboratory.
• **Key Results** Free-living and lichenized forms of *Prasiola* were identified as two different species: *P. crispa* and *Prasiola* sp., respectively. While lichenization appears to have no effect on the photochemical performance of the alga or its tolerance of desiccation (in the short term), the symbiotic lifestyle involves (1) changes in water relations, (2) a considerable decrease in the net carbon balance and (3) enhanced freezing tolerance.
• **Conclusions** Our results support improved tolerance of sub-zero temperature as the main benefit of lichenization for the photobiont, but highlight that lichenization represents a delicate equilibrium between a mutualistic and a less reciprocal relationship. In a warmer climate scenario, the spread of the free-living *Prasiola* to the detriment of the lichen form would be likely, with unknown consequences for Maritime Antarctic ecosystems.

Key words: Abiotic stress, alga, desiccation tolerance, freezing tolerance, glassy state, lichen, *Mastodia tessellata*, photobiont, photoprotection, photosynthesis, polar, *Turgidosculum*.

INTRODUCTION

Lichens are symbiotic organisms resulting from the association of one fungal and at least one photosynthetic partner (mycobiont and photobiont, respectively). They can thrive in some of the harshest environments on Earth, such as drylands and tundra (e.g. Antarctica). As an illustrative example of their relevance, the current Antarctic flora includes more than 350 species of lichens, around 130 species of bryophytes (100–115 mosses and 27 liverworts) and only two species of tracheophytes (Kappen, 2000; Øvstedal and Lewis Smith, 2001; Peat *et al.*, 2007; Ochyra *et al.*, 2008). Lichens play essential roles in primary production and nutrient cycling in diverse ecosystems (Asplund and Wardle, 2017), including polar regions, where they represent a large fraction of the local biodiversity. Hence, the understanding of the ecophysiology of lichens as primary producers is of crucial relevance. With regard to climate change scenarios, lichens receive increasing attention, with particular emphasis on more vulnerable environments such as drylands and polar habitats (Laguna-Defior *et al.*, 2016; Bao *et al.*, 2018; Colesie *et al.*, 2018; Ladrón de Guevara *et al.*, 2018). In this sense, different manipulative

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experiments have recently indicated that: (1) increasing temperatures may alter lichen communities in drylands (Ladrón de Guevara *et al.*, 2018) and the Antarctic (Colesie *et al.*, 2018), (2) a higher frequency of freezing/thawing events during winter may have a negative impact on Arctic and Antarctic lichens (Harańczyk *et al.*, 2003; Bjerke, 2011), and (3) a prolonged period under snow may threat lichen subsistence in Maritime Antarctica (Bokhorst *et al.*, 2016; Sancho *et al.*, 2017).

Lichens are poikilohydric organisms and, the vast majority of the species are considered to be tolerant of desiccation, i.e. able to withstand extremely low cellular water contents [<0.1 g H_2O g^{-1} dry weight (DW)] and to reactivate metabolic activity upon rehydration (Kranner *et al.*, 2008). Thus, very finely tuned physiological and biochemical processes, related mainly to photochemical and antioxidant protection, seem to be key aspects of their capacity to deal with severe cell dehydration (Kranner, 2002; Heber *et al.*, 2007; Fernández-Marín *et al.*, 2010). In dry lichens, a particular quenching of chlorophyll (Chl) fluorescence relates to the efficient dissipation of light energy during dry periods (Veerman *et al.*, 2007; Heber *et al.*, 2010). Among antioxidants, zeaxanthin and glutathione seem to play a major role in the preservation of chloroplast and cell functionality during desiccation/rehydration cycles (Kranner *et al.*, 2003; Fernández-Marín *et al.*, 2010). Only recently, the loss of cell turgor has been pointed out as a relevant sensor and a trigger of desiccation-tolerance responses (Banchi *et al.*, 2018), and the physiology of their water relations is just starting to be addressed from an irreversible thermodynamics approach, e.g. by measuring water potential isotherms (Nardini *et al.*, 2013; Petruzzellis *et al.*, 2018). Other physiochemical consequences of desiccation, such as entering into the so-called glassy state, which severely limits biochemical reactions in desiccation-tolerant mosses and tracheophytes (Fernández-Marín *et al.*, 2013, 2018*b*), remain unexplored in lichens so far.

Albeit lichens dominate cold environments and although desiccation and low temperatures lead to many similar physiological consequences in photosynthetic cells (Fernández-Marín *et al.*, 2018*b*; Verhoeven *et al.*, 2018), their tolerance of freezing has been greatly understudied in lichens in comparison with other organisms (Bjerke, 2011; Nowak *et al.*, 2018). This may be because (1) as poikilohydric organisms lichens are assumed to withstand low temperatures when dry (Gauslaa and Solhaug, 1999) and (2) some species (from boreo-alpine and polar regions, mainly) are capable of C assimilation at sub-zero temperatures (Kappen and Lange, 1972; Kappen, 1989; Barták *et al.*, 2007). Recent work, however, has shown great variability in the sensitivity of lichen species to freezing when wet and have evidenced an important gap in our knowledge regarding the physiological and molecular aspects involved in it (Bjerke, 2011; Hájek *et al.*, 2016; Nowak *et al.*, 2018; Solhaug *et al.*, 2018). Although the molecular mechanisms of frost and desiccation tolerances are not fully understood in lichens (Nowak *et al.*, 2018), several studies have pointed out that the symbiosis is beneficial to the photobiont by counteracting desiccation (Kranner *et al.*, 2005; Kosugi *et al.*, 2009), while others have highlighted the capability of the free-living photobiont to withstand low temperature and/or desiccation, particularly within the genus *Trebouxia* (Sadowsky and Ott, 2012; Candotto Carniel *et al.*, 2015).

Overall, the complex relationships among the components of the lichen consortium hinder the understanding of their separate physiologies and thus of the physiological consequences of the symbiosis for the individual partners. Until now, major advances in their understanding are a result of studying isolated symbionts cultured under laboratory conditions. Valuable information has been obtained through this approach regarding different aspects of photobiont ecophysiology, e.g. cell osmolarity (Kosugi *et al.*, 2014), nutrient uptake (Pavlova *et al.*, 2017), photochemistry (Guéra *et al.*, 2016) and, more recently, water relations (Centeno *et al.*, 2016; Banchi *et al.*, 2018). Field studies would still be needed to completely understand the ecophysiology of lichen photobionts in their free-living forms. Nevertheless, their sparse presence in the environment and the fact that they usually cohabit with many other taxa of microflora [e.g. on the surface of other lichen species' thalli (Muggia *et al.*, 2013)] make the study of free-living photobionts an extremely challenging task under natural conditions.

A unique opportunity, though, arises from the study of the exceptional association established by the lichen-forming fungus *Mastodia tessellata* (Verrucariaceae) and its photobiont, a green alga of the genus *Prasiola* (Prasiolales, Trebouxiophyceae). Two main reasons for this are (1) the macroscopic and multicellular nature of the photobiont, the only known case, together with *Turgidosculum ulvae*, of a fungus forming a lichen symbiosis with a blade-like alga (Pérez-Ortega *et al.*, 2018), and (2) the apparent coexistence of free-living and lichenized forms of *Prasiola* in the same microenvironments (Huiskes *et al.*, 1997*a*) (see also Fig. 1). The characteristic symbiosis formed by *M. tessellata* has repeatedly attracted the attention of the scientific community. The species occurs in the upper littoral zone at high latitudes of both hemispheres, with extant populations in Alaska (USA), British Columbia (Canada), Tierra del Fuego (Chile), New Zealand, Tasmania (Australia), Antarctica and the sub-Antarctic islands (for a more detailed distribution area see Garrido-Benavent *et al.*, 2018). Pérez-Ortega *et al.* (2010) studied the phylogenetic affinities of both symbionts, and dismantled the monotypic family Mastodiaceae, showing that *M. tessellata* is closely related to marine members of the family Verrucariaceae. These authors also thoroughly characterized the interactions between the two symbionts at the ultrastructural level, revealing a particular kind of fungal haustorium not previously observed, and pointing to a relationship that was not parasitic but rather a dynamic equilibrium in which the photobiont can occasionally escape from the mycobiont. More recently, it has been shown that there are more than one species of *Prasiola* algae forming lichen symbioses with *M. tessellata* (Garrido-Benavent *et al.*, 2017). The known bipolar distribution of the lichen-forming fungus and its photobionts is the result of a complex of past events, with relatively recent joint dispersal from the southern hemisphere to the northern hemisphere (Garrido-Benavent *et al.*, 2018).

To the best of our knowledge, *in situ* comparison (in Antarctica) of free-living versus lichenized *Prasiola* (e.g. *M. tessellata*) has only been done before by Huiskes *et al.* (1997*a*). Huiskes and co-workers showed that subjection to prolonged hydration was detrimental to the photosynthetic rates of the lichen but not of the free-living *Prasiola* specimens (Huiskes *et al.*, 1997*a*). With a broad combination of methodologies and by integrating

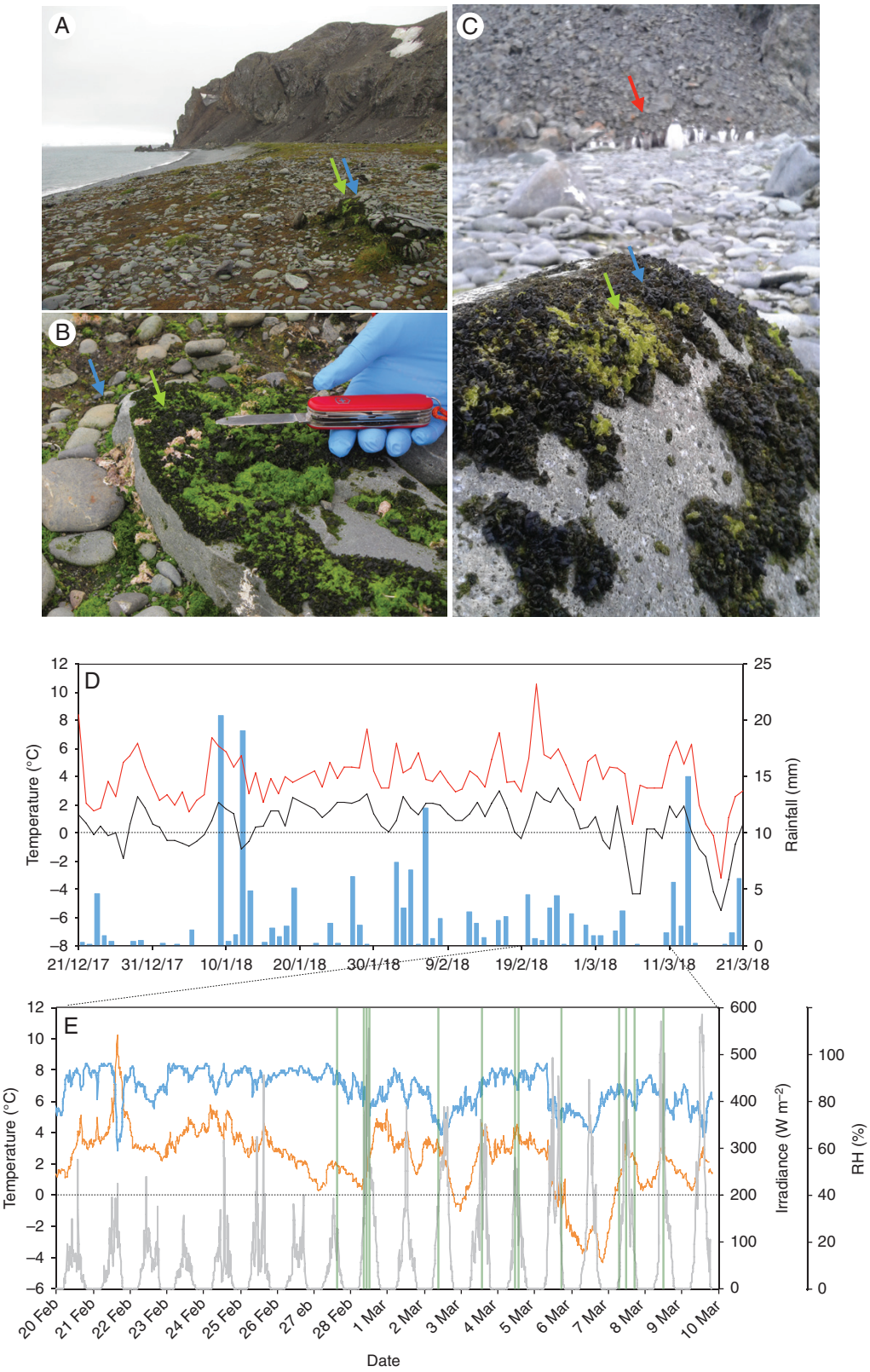

FIG. 1. Collection site: characterization and meteorology. (A) Field site: Argentina Cove in Livingston Island (Maritime Antarctica). (B) Detail of the micro-environment showing the intricate patched carpet formed by *M. tessellata* and *P. crispa*. (C) Gentoo penguin colony (red arrow) close to the studied populations. In panels (A), (B) and (C), blue arrows indicate *M. tessellata* and green arrows indicate *P. crispa*. (D) Meteorological data for the summer of the study obtained from the BAE JCI station: maximum and minimum day temperatures are depicted in red and black, respectively. Blue bars depict cumulative day precipitation. (E) Meteorological data for a temporal window of 1 month including the experimental days (23 February to 6 March). Relative humidity, average temperature and irradiance are presented as blue, orange lines and grey lines, respectively. Vertical green lines highlight the measuring time points for YII (see the Materials and methods section for further details).

in situ and *ex situ* measurements, we aimed to address three aspects of photobiont physiology: (1) differences in photosynthetic performance of co-occurring populations of free-living and lichenized *Prasiola*; (2) the extent to which the symbiosis influences water relations in *Prasiola*, including its tolerance of desiccation; and (3) how the association affects survival and performance of *Prasiola* in sub-zero temperatures.

MATERIALS AND METHODS

Field site

Co-occurring samples of lichenized and free-living *Prasiola* (Fig. 1A–C) were measured and/or collected on Livingston Island, South Shetland Islands (62º40′ S, 60º23′ W), near the Spanish Antarctic Research Station Base Antártica Española Juan Carlos I' (BAE JCI) (for further details of the base's location see Laguna-Defior *et al.*, 2016) during summer 2018 (23 February to 6 March). Both *Prasiola* forms were growing on a rocky substrate in Argentina Cove, close to a gentoo penguin (*Pygoscelis papua*) colony (Fig. 1C). Intermediate forms between completely free-living *Prasiola* and the lichen *Mastodia tessellata*, as described by other authors (Huiskes *et al.*, 1997*b*; Pérez-Ortega *et al.*, 2010), were observed but avoided during field measurements and collections. Measurements took place in austral summer 2017–18, and the weather was characterized by a mean air temperature of 2.3 °C, a range of variation between 10.6 and −5.5 °C, and a small daily thermal amplitude (3.3 °C) (Fig. 1D). Freezing temperatures only occurred at the beginning and end of the season, and were particularly noticeable in March. Rainfall totalled 173 mm during the three summer months (21 December to 21 March), and there was no rain on only one-third of the days (<0.1 mm) (Fig. 1D). The study period (25 February to 10 March) was characterized by two contrasting situations (Fig. 1E). The first week was pretty much comparable to the average summer trend (mean air temperature 2.5 °C, humid and cloudy), while the second week was colder (0.7 °C) and there were many clear sunny periods (Fig. 1E). All meteorological data were kindly provided by the Spanish Agency of Meteorology (AEMET).

Identity of free-living and lichenized Prasiola

Identification of *Prasiola* specimens is usually not a straightforward task due to the reduced number of available taxonomic characters and the presence of cryptic species (Moniz *et al.*, 2012). We used a fragment of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L), which has been used in recent studies to infer phylogenetic relationships in the genus (Moniz *et al.*, 2012) and to reconstruct the complex evolutionary history of photobionts associated with *M. tessellata* (Garrido-Benavent *et al.*, 2017). Ten samples (five lichenized, five free-living) were analysed for molecular identification. A small fragment (~1 mm^2) of sample, cleaned under a dissecting microscope to avoid epiphytic algae, was removed with a razor blade and checked under the light microscope. DNA extraction was carried out using the E.Z.N.A.® Forensic Kit (VWR, Spain) following the manufacturer's instructions. Amplification was carried out using the primer pair *rbc*L-pras-F and *rbc*L-pras-R (Garrido-Benavent *et al.* 2018). PCR reactions were performed in a total volume of 15 µL, containing 2 µL of template DNA, 0.5 µL of each primer (10 µM) and 6.5 µL of MyTaq Mix [MyTaq DNA Polymerase (Bioline) and dNTPs]; distilled water was added to reach the final volume. PCR amplifications were performed in an Eppendorf Mastercycler EP gradient S thermal cycler. PCR conditions followed Garrido-Benavent *et al.* (2018). PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher) according to the manufacturer's instructions and sequenced by Macrogen Inc. (Madrid, Spain) using the same primer sets as for PCR amplification. Sequence contigs were assembled using SeqMan v.14 (Lasergene, DNA Star, WI, USA).

Experimental design

The combination of physiological measurements in the field with physicochemical and biochemical analyses in the BAE JCI and University of the Basque Country (UPV/EHU) laboratories allowed an in-depth ecophysiological comparison of free-living and lichenized forms of *Prasiola*. Alongside the aims of the work, three main experiments were conducted.

Experiment 1. To evaluate the differences in photosynthetic performance of co-occurring populations of lichenized versus free-living specimens of Prasiola we combined (1) analyses of photochemical performance and photosynthetic pigment composition under natural conditions in the field with (2) gas exchange measurements of freshly collected wet samples under controlled conditions in the BAE JCI laboratories (Figs 2–4). The assessment of photochemical and photosynthetic pigment adjustments in response to natural irradiance was conducted in situ in intact thalli of both species in their natural microenvironment. The actual yield (YII) of photosystem II (PSII), sample temperature and photosynthetic photon flux density (PPFD) were recorded in the field under diverse irradiance conditions and the samples were preserved (fast-dried as described in Esteban et al., 2009) for later analysis of photosynthetic pigments. Three measurements per species and time-point were conducted at a total of 23 time-points with different PPFDs during the days 27 February, 26 February, 2 March, 3 March, 4 March, 5 March, 7 March and 8 March (Fig. 1E, green lines). Further details of chlorophyll fluorescence, gas exchange and pigment analyses are explained below in specific subsections of the Materials and methods section. Experiment 2. This experiment was conducted to evaluate the influence of the symbiosis in water relations of *Prasiola*, including its tolerance of desiccation. For this purpose, pressure–volume (P–V) curves and a desiccation experiment were conducted at the BAE JCI, and an additional set of samples was collected, dried with silica gel and used to assess glass transition temperature (T_g) and hydrophobicity of thallus surfaces in the UPV/EHU laboratories (Figs 5 and 6). The desiccation experiment was performed by equilibrating freshly collected free-living and lichenized samples at 10, 50, 80 and 100 % relative humidity (RH) for 48 h in darkness at natural outdoor temperatures (~ +4 ºC, data from AEMET station at the BAE JCI for the days of the experiment, 2–4 March 2018; Fig. 1). Using an adaptation of the method

described in López-Pozo *et al.* (2019), incubations were performed in 1-L volume hermetic boxes containing 200 mL of silica gel (to give 10 % RH), $MgCl_2$ solution (1.87 g of $MgCl_2$ hexahydrate per mL of Milli-Q water, to get 50 % RH), NaCl saturated solution (80 % RH) or distilled water (100 % RH). The equilibrium RH within the hermetic boxes was tested with an RH and temperature data logger (EL-USB- 2-LCD+; Lascar Electronics, UK). Solutions were adsorbed on a commercial kitchen sponge (Spontex Natura®) separated from the samples by a plastic mesh, and the samples were placed over Petri dishes so that direct contact with the solutions was prevented. Boxes were covered with aluminium foil (to ensure darkness) and located outdoors in the shade to prevent overheating by direct sun exposure. Incubation was conducted for 48 h. To assess the changes in photosynthetic pigments induced by the different incubations, samples were collected after 48 h and immediately frozen in liquid nitrogen. Maximal photochemical efficiency of PSII (F_v/F_m) was monitored before incubation and after 48 h. Additionally, a separate set of samples desiccated at 50 % RH were monitored during rehydration at 0, 2, 5, 10, 20, 30 min and 1, 6, 12, 24 and 48 h for detailed analysis of F_v/F_m recovery. A last set of silica-gel dried samples was preserved for further analyses at the UPV/EHU: P–V curves, T_g estimation and evaluation of thallus hydrophobicity. Detailed information on these measurements is given in specific subsections of the Materials and methods section.

Experiment 3. In order to assess how the lichen symbiosis affects the survival and performance of *Prasiola* at sub-zero temperatures, a controlled freezing experiment was conducted in the BAE JCI laboratories and a second sample set was used for the assessment of freezing temperature at the UPV/EHU laboratories (Fig. 7). For the freezing experiment, freshly collected wet samples were acclimated to darkness for 12 h and subsequently incubated at −20 ºC for 48 h. Changes in photosynthetic pigments were analysed before and after freezing, and F_v/F_m was monitored during incubation at −20 ºC and during re-warming at +20 ºC. The experiment was performed in darkness. Further details of the assessment of ice nucleation temperature are given later in a specific subsection of the Materials and methods section.

Whenever required in any of the experiments, controlled hydration was conducted with distilled water since previous studies showed that *P. crispa* and *M. tessellata* are salt-tolerant but not obligate halophytes, being undamaged by hypo-osmotic water (Jacob *et al.*, 1991; Smith and Gremmen, 2001*a*).

Chlorophyll fluorescence

Two types of measurements were conducted with a portable modulated Plant Stress Kit Fluorometer (PSKF, Opti-Sciences, Hudson, NH, USA): YII measurements in the field and F_v/F_m in the BAE JCI laboratory. The maximum Chl *a* fluorescence under solar radiation (F_m') was induced with a saturating pulse of 0.8 s and 7000 µmol m^{-2} s^{-1}. The actual efficiency of PSII (YII) was estimated as YII = $(F_m' - F_s)/F_m'$, where F_s is the actual Chl *a* fluorescence under solar illumination. The saturation pulses for F_m' estimation were applied as described by Loriaux *et al.* (2013). Maximal photochemical efficiency of PSII (F_v/F_m) was assessed in dark-adapted samples in the laboratory for Experiments 2 and 3.

Gas exchange measurements

Net assimilation of CO_2 (A_n) was determined by using a GFS-3000 gas exchange system coupled with a fluorometer (IMAGING-PAM; Heinz Walz, Effeltrich, Germany). The blue light source provided by the manufacturer, with maximum emission at 420 nm, was used during fluorescence and gas exchange measurements. Fully hydrated samples (n = 4 or 5) were introduced in a custom-made cuvette consisting of a gasket stuck to a thin piece of polyester cloth. The size of the gasket used equalled that of the chamber, so that proper closure of the chamber was possible and CO_2 leakage was close to zero. A similar sample size was used for all replicates of *M. tessellata* and *P. crispa* (average ± s.e. 3.6 ± 0.3 and 3.6 ± 0.2 cm^{-2}, respectively). Fresh weight (FW) and DW were individually recorded for each replicate (*M. tessellata* 77 ± 5 and *P. crispa* 16 ± 2 mg DW on average). The CO_2 concentration was standardized at 400 p.p.m. and RH at 75–85 %, and the flow rate within the chamber was 750 µmol s^{-1}. Sample temperature was set to +20 ºC, which has previously shown to be optimal for maximum CO_2 assimilation rate in *M. tessellata* (Smith and Gremmen, 2001*b*). We recorded A_n under steady-state conditions when diffusion limitations due to interstitial water were null and the biochemistry was fully adapted to light–temperature (3–5 min). Ten light intensities (0, 20, 40, 75, 165, 370, 580, 922, 1465 and 1855 µmol m^{-2} s^{-1}) were set in order to obtain a light curve. If needed, full rehydration of the sample during the light curve was done in order to avoid its desiccation by immersing it in distilled water for 1–2 min and gently removing the excess water with a paper tissue before storing the sample in the cuvette again. The negative A_n at 0 of irradiance (I) was considered the dark respiration value (R_d). The maximum light-saturated A_n (A_{sat}) and the apparent quantum efficiency (AQE) were obtained by fitting each light curve to Michaelis–Menten (Ritchie and Prvan, 1996*a*, *b*), rational (Smith, 1936), exponential (Goudriaan, 1982) or hyperbolic tangent (Jassby *et al.*, 1976) models by using the Microsoft Excel Solver tool (Lobo *et al.*, 2013). The lowest square sum errors were obtained with the Michaelis–Menten model (eqn 1).

$$A_n = \frac{A_{sat} \cdot I}{A_{sat}/AQE \ + I} - R_d \quad (1)$$

Calculation of the light compensation point at which A_n equals zero (I_{com}) and the irradiance at which 90 % of the A_{sat} is achieved (I_{sat90}) was done for the Michaelis–Menten model (eqns 2 and 3).

$$I_{com} = \frac{A_{sat} \cdot R_d}{AQE(A_{sat} - R_d)} \quad (2)$$

$$I_{sat90} = \frac{A_{sat}(9A_{sat} + R_d)}{AQE \cdot A_{sat} \cdot R_d} \quad (3)$$

Photosynthetic pigment and tocopherol analysis

Photosynthetic pigments and tocopherols were analysed by HPLC following the method described by García-Plazaola and Becerril (1999, 2001) with small modifications. Briefly, ~10 mg DW of thalli was used per replicate and three or four

replicates per treatment and/or time-point for photosynthetic pigment analysis. Samples were immediately frozen in liquid nitrogen in the field after treatment and freeze-dried before analysis. Samples were doubly extracted in 0.25 mL of cold (+4 °C) acetone:water (95:5 v:v) first, and in 0.25 mL of pure acetone second. All acetone solutions were buffered with $CaCO_3$. Both supernatants were filtered through a 0.2-µm polytetrafluoroethylene filter (Teknokroma, Barcelona, Spain) before being analysed by HPLC. Tocopherol and pigment detection and quantification were conducted with a scanning fluorescence detector (Waters 474) operating in series with a photodiode array detector (Waters 996; Waters, Milford, MA, USA) (García-Plazaola and Becerril, 1999, 2001). The relative de-epoxidation state of the xanthophyll cycle pigments was estimated as the ratio (antheraxanthin + zeaxanthin)/(violaxanthin + antheraxanthin + zeaxanthin), abbreviated as AZ/VAZ.

Water potential measurements

Sample water potentials were determined with a dewpoint hygrometer (WP4; Decagon Devices, Pullmann, WA, USA) in the laboratory of the BAE JCI, following a protocol similar to that previously used for bryophytes and lichens (Proctor *et al.*, 1998; Nardini *et al.*, 2013; Petruzzellis *et al.*, 2018). The instrument was calibrated at the beginning of each measurement session using 0.5 M KCl standard solution. The fully hydrated thalli (equilibrated for 24 h at 100 % RH over wet paper) were measured for their initial water potential (Ψ) and immediately weighed to determine the initial fresh weight. Samples were left to dry on the laboratory bench at 50 % RH and 22–24 ºC, and Ψ was measured subsequently at different times during dehydration. After each reading, samples were immediately weighed to match each Ψ value to the corresponding weight. At the end of the measurements, each sample was dried in the oven at 70 ºC for 24 h to determine the DW. Absolute and relative water contents (WC, RWC) were then calculated for each sample and time point.

Typical P–V curves were obtained by plotting the 1 − RWC values (*x*-axis) against the corresponding $-1/\Psi$ (*y*-axis). Two phases were distinguished during desiccation of samples: in the first phase, small changes in Ψ occurred with high losses of water from the samples until the turgor loss point (TLP) was reached. In a second phase, which corresponds to values below the TLP, the curve became linear ($r^2 > 0.98$). The *y*-axis intercept of the linear portion of the P–V curve is the reciprocal of the full-turgor water potential (Ψ_o). The point at which the linear portion of the curve begins to curve upwards towards the *y*-axis is the TLP; the points between TLP and the *y*-axis provide the data for calculating cell wall elasticity, known as the modulus of elasticity (ε). The following parameters were calculated according to Sack *et al.* (2011): water content at full turgor (saturated water content, SWC, g H_2O g^{-1} DW) Capacitance before turgor loss (C_{FT}) and at the TLP (RWC_{TLP}, %); osmotic potential at full turgor (Ψ_o, MPa); water potential at the TLP (Ψ_{TLP}, MPa); and modulus of elasticity (ε, MPa). Specifically, the water mass at turgor was estimated as the intercept of sample water mass and Ψ at which Ψ equals zero. Then, the SWC was calculated as water mass at turgor/DW, and one value was obtained per sample. The RWC was estimated as (FW − DW)/(TW − DW) − 100, where TW − DW is water mass at turgor, and several data points were obtained for each sample during the desiccation process.

Molecular mobility

Mechanical analyses were conducted on desiccated thalli (equilibrated with silica gel) of lichenized and free-living *Prasiola* in order to estimate how lichenization affects molecular mobility of dry thalli. These measurements provide information about the extent of molecular mobility (and thus the potential enzymatic activity) at a wide range of temperatures (Ballesteros and Walters, 2011). A dynamic mechanical thermal analyser (dma/sdta861e, Mettler Toledo, Greifensee, Switzerland) in the shear mode was used for the mechanical analysis. The method was similar to that described by Fernández-Marín *et al.* (2013) with the modifications described by Fernández-Marín *et al.* (2018*b*). Briefly, measurements from −50 to +150 °C at a heating rate of 2 °C min^{-1} were carried out in the dynamic mode. Each sample was scanned twice. Three biological replicates, of ~300 mg DW each, were measured per species. Shear storage modulus (G'), shear loss modulus (G'') and the loss tangent ($\tan\delta = G''/G'$) were calculated using the Mettler Toledo START software during dynamic mechanical thermal analysis (DMTA) scans. For each biological replicate, the temperature value at the maximum tanδ coincident with the α-relaxation measured at 1 Hz was considered for T_g estimation.

Hydrophobicity of the thallus surface

The contact angle of droplets of distilled water was measured over thalli equilibrated with silica gel in order to estimate the hydrophobicity of their surface, following a method similar to that previously used for lichens (Hauck *et al.*, 2008). Measurements were conducted with a video-based optical contact angle measuring instrument (OCA 15EC; DataPhysics Instruments, Filderstadt, Germany). Sessile droplets of 3 µL were placed on the upper side of the thalli. The contact angle was estimated with the SCA software for optical contact angle, v.4.4.3. The measuring precision was ±0.1º. Measurements were performed on ten thalli per species at 1 s after contact. Each value was the mean from the optically left and right margins of the water droplet.

Determination of ice nucleation (freezing) temperature

The freezing temperature of lichenized and free-living *Prasiola* samples was analysed with a differential scanning calorimeter (DSC 822e; Mettler Toledo, Greifensee, Switzerland) at full turgor in thalli of ~10 mg, following a method similar to that described by Fernández-Marín *et al.* (2018*b*). Full-turgor samples were sealed in aluminium pans and characterized under constant liquid nitrogen flow (20 mL min^{-1}). First, samples were equilibrated at 0 °C and then cooled at a rate of 0.1 °C min^{-1} from 0 to –15 °C. Subsamples of the same set as that used for DSC scans were used to estimate absolute water

content. Before the measurements, the DSC equipment was calibrated with zinc, indium and pure water as standards. All weights were recorded using a Mettler Toledo 0.1-mg precision balance. DSC measurements were performed on at least five thalli per species.

Statistics

Normal distribution of data was checked with the Kolmogorov–Smirnov test and homogeneity of variances with the Levene test. One-way ANOVA was used to test for differences between lichenized and free-living forms of *Prasiola* in each of the analysed parameters: pigment contents, WC, F_v/F_m, T_g and freezing temperature, contact angle, and parameters derived from P–V curves and gas exchange modelling. When data showed heteroscedasticity, the non-parametric Kruskal–Wallis test was used. Statistical analyses were conducted with SPSS v24.0 and significance was accepted at $\alpha = 0.05$.

RESULTS

Identification of Prasiola *species*

Sequences recovered from free-living and lichenized co-occurring samples of *Prasiola* in Argentina Cove showed that they corresponded to two different species. All sequences gathered from lichenized *Prasiola* were identical, and in BLAST searches (Altschul *et al.*, 1997) 100 % matched *Prasiola* sp., a putative new species previously reported from other lichenized *Prasiola* samples from Antarctica (Garrido-Benavent *et al.*, 2017). Sequences from free-living specimens were almost identical (differing at only five positions) and all matched either 100 or 99 % with *Prasiola crispa* in BLAST searches.

Photochemistry

Figure 1E shows the meteorological conditions (RH, temperature and, more importantly, irradiance) during the measurements of YII and the collection of samples for the subsequent analyses of photosynthetic pigments and tocopherols. The extent of the study period allowed us to get a detailed picture of the response of photochemical parameters to the environmental constraints, e.g. a wide range of irradiances was covered. Figure 2 shows the average content of photosynthetic pigments and tocopherol of samples that were directly preserved in liquid nitrogen in the field. Lichenized and free-living *Prasiola* did not differ qualitatively in their pigment and tocopherol composition. All major carotenoids typical of green photosynthetic organisms, including α-carotene (α-Car), not always present, were found, and α-tocopherol (α-Toc) was the only isoform of tocopherol identified in both species. Nevertheless, several differences were found in the quantitative contents of photosynthetic pigments. The free-living *P. crispa* showed a significantly higher content of Chl when expressed in terms of DW, and a significantly lower Chl *a*/*b* ratio (Fig. 2A, B). The free-living

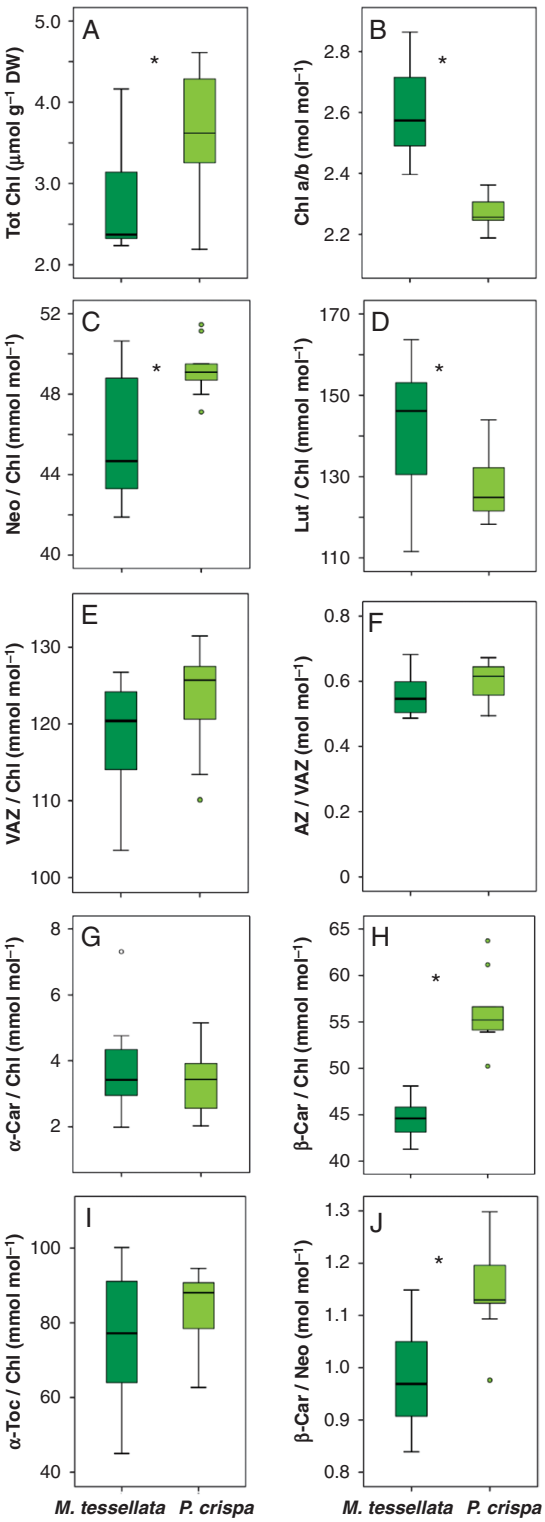

FIG. 2. Boxplots showing the contents of photosynthetic pigment and tocopherol in *M. tessellata* and *P. crispa* thalli collected directly in the field. Boxes cover 50 % of the data; central lines represent the medians and whiskers represent the minimum and maximum values among non-atypical data. Open circles represent outliers. The numbers of reported data are $n = 8$ for *M. tessellata* and $n = 9$ for *P. crispa*. Asterisks denote significant differences ($P < 0.05$) between the two species (one-way ANOVA).

P. crispa also showed lower thallus mass area (TMA) than *M. tessellata* (45 ± 4 versus 224 ± 27 g m^{-2}, data not shown) and a predominantly mono- or di-stromatic structure with a higher concentration of photosynthetic cells (Supplementary Data Fig. S1). Its lutein (Lut) and β-Car to Chl ratios were higher and its neoxanthin (Neo) to Chl ratio was lower than in the lichenized form (Fig. 2C–H). No significant differences were found in xanthophyll cycle pigments or in α-Toc content between the two species (Fig. 2E, F, I). Interestingly, the de-epoxidation index of the xanthophyll cycle in these field-sampled thalli was always >0.4 for both species.

An exhaustive sampling of actual photochemical efficiency (YII) during 10 d of measurements was conducted *in situ* (Fig. 3; the exact data points are highlighted as green lines in Fig. 1E). This allowed a detailed characterization (n = 122 data points per species) across a good representation of the natural irradiance range occurring in the microenvironment of both *Prasiola* forms: from 22 to 1679 µmol photons m^{-2} s^{-1} (Fig. 3A, B). The variability of YII values, particularly at lower irradiances, reflects the potential range displayed for both species as depending, for instance, on water availability (see also Fig. 3C, D). Light responses were photochemically similar between the two species, which showed saturation PPFD at around 600 µmol photons m^{-2} s^{-1}. Interestingly, free-living *P. crispa* showed higher YII than the lichenized form *Prasiola* sp. at low PPFD, below ~200 µmol photons m^{-2} s^{-1} (Fig. 3A, B). The trend of photosynthetic pigments in response to last-week precipitation and cumulative irradiance was also evaluated (Fig. 3C, D). While the two species responded similarly, there was a marked (although not significant) increasing trend in AZ/VAZ in response to higher cumulative irradiance (Fig. 3C) and a decreasing trend in response to higher cumulative precipitation (Fig. 3D).

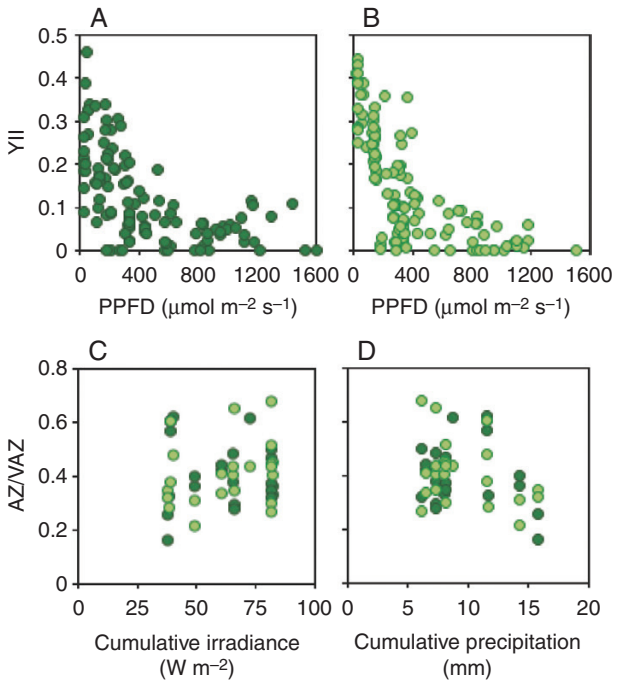

FIG. 3. Photochemical parameters measured in the field under natural irradiance (PPFD) for *M. tessellata* (dark green) and free-living *P. crispa* (light green). Upper panels show the response of actual photochemical efficiency of PSII (YII) to instantaneous irradiance over the thalli of (A) *M. tessellata* and (B) *P. crispa*. Data points are individual measurements (n = 122 per species). Lower panels show changes in the de-epoxidation index of the xanthophyll cycle in response to the last week's cumulative irradiance (C) and precipitation (D). Data points are individual measurements (n = 23 per species).

Gas exchange

The value of A_{sat} was 47.1 % higher in the free-living alga (Fig. 4B), as a result of increased dark respiration due to the fungal contribution in the lichen (R_d of 1.61 ± 0.12 µmol CO_2 m^{-2} s^{-1}, mean ± s.e.), than in the free-living *P. crispa* (0.47 ± 0.0712 µmol CO_2 m^{-2} s^{-1}) (Fig. 4C). Net carbon assimilation in response to irradiance was very similar in the two species on a thallus area basis (Fig. 4). By contrast, due to dissimilar TMA and Chl content of the two species (see above), the differences in gas exchange values were accentuated when expressed on a biomass basis (Supplementary Data Figs S2 and S3). Thus, A_{sat} was 7-fold higher in *P. crispa* than in *M. tessellata* on a Chl basis (Supplementary Data Fig. S3B) and 9-fold higher on a DW basis (Supplementary Data Fig. S2B). The value of R_d (on a Chl basis) was not significantly different between the two species (Supplementary Data Fig. S3C) but was higher in *P. crispa* when expressed on a DW basis (Supplementary Data Fig. S2C). The value of I_{com} was significantly higher in the lichen (78.5 ± 11.9 compared with 12.8 ± 2.3 µmol photons m^{-2} s^{-1}in *P. crispa*; Fig. 4E) and I_{sat90} was accordingly achieved at higher irradiances (Fig. 4D). The two species showed an equal AQE (data not shown). A similar trend occurred when light curves were produced at +10 ºC, with an expected displacement towards lower I_{com} and I_{sat90} (Supplementary Data Fig. S4).

Water relations and tolerance of desiccation

Pressure–volume curves and corresponding estimated parameters for thalli of *M. tessellata* and *P. crispa* are shown in Fig. 5. Both osmotic and water potential at turgor were lower in *M. tessellata* than in *P. crispa*: Ψ_o = −1.9 ± 0.8 versus −0.9 ± 0.0 MPa; and Ψ_{TLP} = −3.6 ± 0.1 versus −2.6 ± 0.1 MPa, respectively (Fig. 5C, D). Interestingly, the water content at saturation in *P. crispa* (SWC = 2.7 ± 0.0 g H_2O g^{-1} DW) was 2-fold higher than in *M. tessellata* (1.4 ± 0.10 g H_2O g^{-1} DW; Fig. 5E). The modulus of elasticity was also very distinct between the two species, being 3.5-fold higher in *M. tessellata* (3.8 ± 0.0 MPa) than in *P. crispa* (1.1 ± 0.1 MPa; Fig. 5F). Remarkably, the TLP occurred at significantly higher RWC in *M. tessellata* (RWC_{TLP} = 60 ± 1 %) than in *P. crispa* (42 ± 1 %; Fig. 5G). Capacitance at full turgor (C_{FT}) was significantly higher in *M. tessellata*.

Further parameters related to water relations and the capacity to counteract cell dehydration are shown in Fig. 6. Controlled desiccation by equilibrium at three different RHs, in darkness, led to different final water contents (e.g. desiccation extents) of >0.2, around 0.1 and <0.1 g H_2O g^{-1} DW in both species (Fig. 6A). All samples were able to recover their initial F_v/F_m values after 48 h of rehydration, indicating comparable tolerance of short-term (48 h) desiccation in *Prasiola* sp. and *P. crispa* regardless

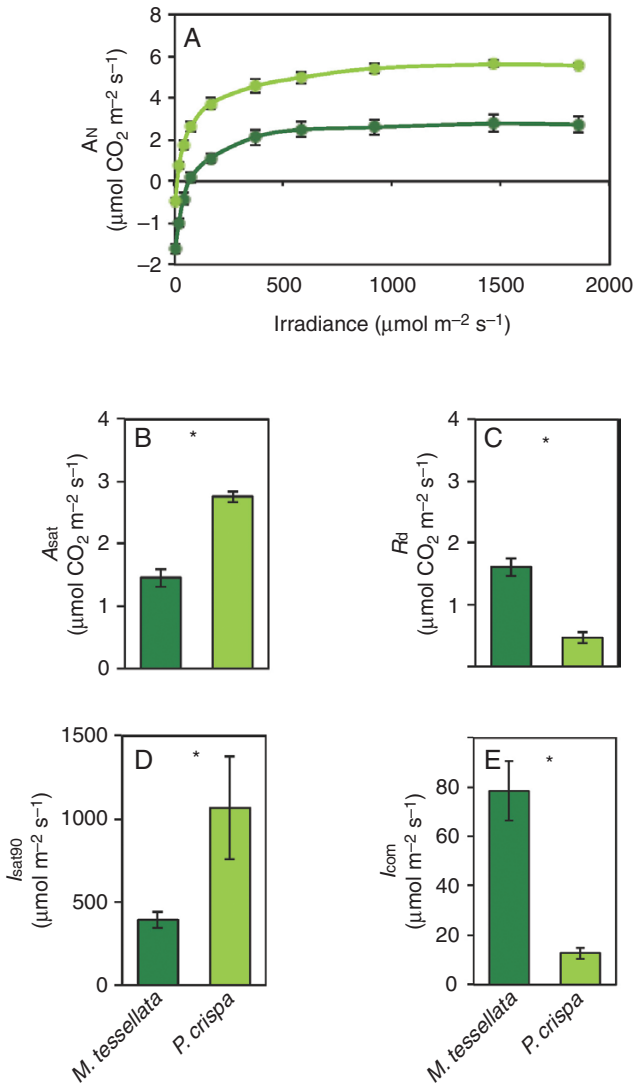

FIG. 4. Gas exchange data for *M. tessellata* (dark green) and *P. crispa* (light green) measured at +20 ºC in hydrated samples in the laboratory of the BAE JCI. (A) Light curve of net CO_2 assimilation expressed on a thallus area basis. (B), (C), (D) and (E) depict maximum net assimilation A_n (A_{sat}), dark respiration (R_d), irradiance at which 90 % of A_{sat} is achieved (I_{sat90}) and light compensation point (I_{com}), respectively, calculated by Michaelis–Menten curve fitting. Data are mean ± s.e. (n = 4 or 5). Asterisks denote significantly different values ($P < 0.05$).

of the severity of dehydration reached (Fig. 6B). Incubation in darkness induced a slight increase in the de-epoxidation level of both species, although the difference was not significant (Fig. 6C). The mechanical analysis of samples equilibrated at 10 % RH revealed that both species were in the glassy state (e.g. with severely reduced molecular mobility) at ambient temperature (e.g. 4 ºC), since their T_g values were one order of magnitude higher (>40 ºC, Fig. 6D). The average T_g was markedly higher in *M. tessellata* than in *P. crispa* (52.2 ± 4.4 and 45.1 ± 1.0 ºC, respectively) but both *Prasiola* species were able to completely recover photochemical efficiency upon rehydration (Fig. 6E). The contact angle of distilled water droplets over *P. crispa* thalli was on average significantly lower than over *M. tessellata*. This was indicative of significantly higher hydrophilicity in the free-living *P. crispa* (Fig. 6F).

Freezing tolerance

The course of F_v/F_m and xanthophyll cycle pigments during a controlled freezing treatment of wet thalli is shown in Figure 7A, B. A slow but progressive decrease in F_v/F_m was observed during the 48 h of incubation at −20 ºC in both species (Fig. 7A). This decrease was remarkably slower in the lichenized *Prasiola* sp., which kept significantly higher values than *P. crispa* during the first 24 h of incubation. In agreement with this, the recovery of F_v/F_m values during re-warming at +20 ºC was also faster in the lichenized *Prasiola* sp., with significantly higher values during the first 30 min. Both species notably recovered their photochemical activity in the extremely short time lapse of 6 h during rewarming to +20 ºC: the percentage of the initial value was 107 ± 2 in the lichenized *Prasiola* sp. and 97 ± 3 in *P. crispa* (average ± s.e.; Fig. 7A). An increasing trend towards higher de-epoxidation levels was obtained in the thalli at the end of the freezing treatment (around 30 % increase in *M. tessellata* and ~10 % in *P. crispa*), although without statistical significance (Fig. 7B). According to the observed behaviour of F_v/F_m, the average ice nucleation temperature was significantly lower in the lichen (–7.1 ± 0.4 ºC) than in the free-living *P. crispa* (−5.1 ± 0.4 ºC; average ± s.e.) (Fig. 7C; see also Supplementary Data Fig. S4). Thalli of both species subjected to −20 ºC in the dry state recovered relatively faster than wet samples during rehydration at +20 ºC, with no significant differences between the species (data not shown).

DISCUSSION

Lichenized and free-living Prasiola *populations co-occur in polar regions*

The identity of the photobiont of *M. tessellata* as *P. crispa* subsp. *antarctica* and the co-occurrence of free-living and lichenized forms of this species in Antarctica have been long-standing tenets (Huiskes *et al.*, 1997*a*, *b*; Lud *et al.*, 2001*b*). Recently Moniz *et al.* (2012) resurrected *P. antarctica* Kützing as an independent species, almost morphologically indistinguishable from *P. crispa* (Lightfoot) Kützing but molecularly distinct. Garrido-Benavent *et al.* (2017) studied lichenized *Prasiola* populations from the northern and southern hemispheres, including Antarctica, and concluded that while Tierra del Fuego and most of the Alaskan specimens belonged to the species *Prasiola borealis*, most lichenized *Prasiola* specimens from Antarctica corresponded to an undescribed species, not known so far in a free-living state. Subsequently, Garrido-Benavent *et al.* (2018) showed in a thorough analysis that *P. borealis* from Tierra del Fuego and *Prasiola* sp. from Antarctica diverged around the middle to the latest Miocene, coinciding with the opening of the Drake Passage. In our study, we found that the two co-occurring *Prasiola* forms belonged to two different species: free-living specimens corresponded to *P. crispa* and lichenized to *Prasiola* sp. The former is a nitrophilic species that usually grows close to penguin rookeries (Graham *et al.*, 2009) and is known from both hemispheres (Moniz *et al.*, 2012). On the other hand, *Prasiola* sp. is, so far, only known in the lichenized form and from Antarctica (Garrido-Benavent *et al.*, 2017, 2018). The fact that co-occurring forms of *Prasiola* do not belong to the same species as previously thought raises the questions of what the

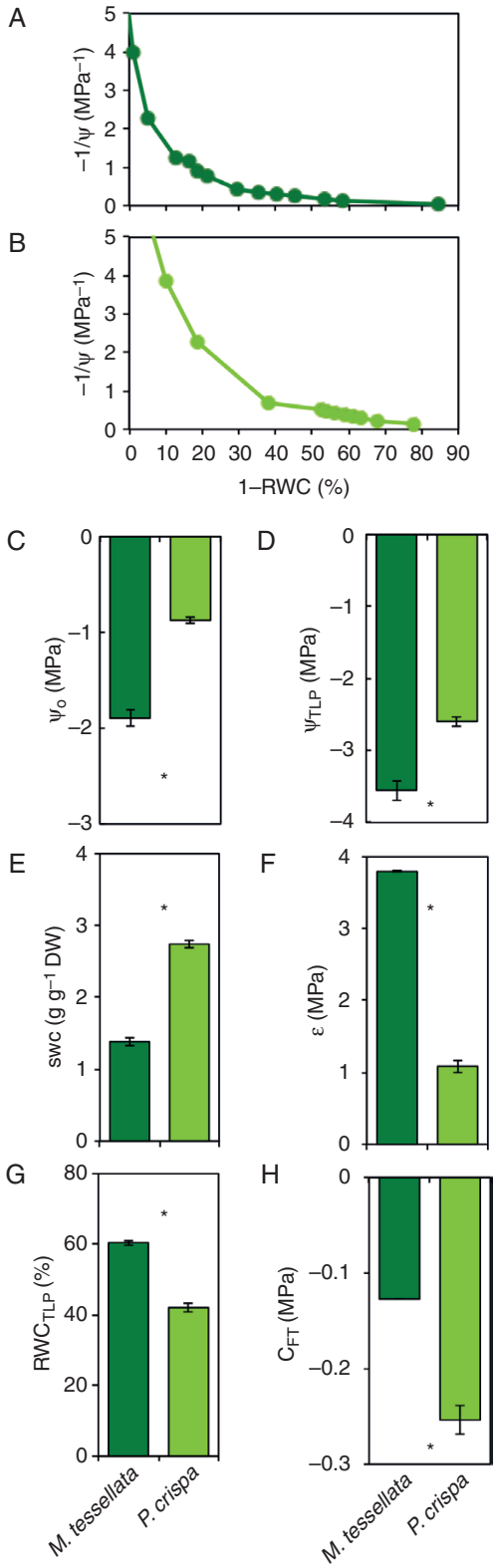

FIG. 5. (A, B) Pressure–volume (P–V) curves of thalli from *M. tessellata* (A) and *P. crispa* (B), showing the changes in inverse water potential ($1/\Psi$) as a function of water loss from the thalli (1 − RWC). One representative measurement is shown for each species. (C–H) Selected parameters estimated from the P–V curves (see Materials and methods section for details of the calculations): water potential at full turgor (Ψ_o), water potential at the turgor loss point (Ψ_{TLP}), symplastic water content at full turgor (SWC), modulus of elasticity (ε), relative water content at turgor loss point (RWC_{TLP}) and capacitance before turgor loss (C_{FT}). Data are mean ± s.e. ($n = 3$, for *P. crispa*, 2 for *M. tessellata*). Asterisks denote significant differences among species at $P < 0.05$.

niche of free-living *Prasiola* sp. is and whether it does really occur in a free-living state. *Prasiola borealis*, the most common photobiont of *M. tessellata* of Antarctica is known to occur in the free-living state (Garrido-Benavent *et al.*, 2017), and so we expect the same is true for *Prasiola* sp., although it may happen that it occupies a different niche when free-living, as is known to be the case for other photobionts (Sanders *et al.*, 2004).

Effect of lichenization on the photosynthetic performance of Prasiola

Both lichenized and free-living specimens showed similar photosynthetic performances, with lower net assimilation rates in the lichenized growth form on a thallus area basis (Fig. 4). This is most likely a result of higher energy consumption and respiratory carbon loss deriving from the mycobiont. Dark respiration rates were 4-fold higher than that of the photobiont in its free-living growth form (Fig. 4A, C). The carbon assimilation of *M. tessellata* and different species of *Prasiola* has been reported previously (Smith and Gremmen, 2001*a*, *b*; Kang *et al.*, 2013; Holzinger *et al.*, 2017). Nevertheless, and as far as we know, photosynthesis data of both *M. tessellata* and *P. crispa* have only been compared by Huiskes *et al.* (1997*b*) so far, at non-saturating irradiation and low temperature. In these conditions, *P. crispa* also showed higher A_n than *M. tessellata*. Photosynthetic capacity of *M. tessellata* was unaffected by low temperatures (from +5 to +20 ºC), while R_d and I_{com} decreased (Smith and Gremmen, 2001*a*, *b*). Although A_{sat} and I_{sat90} decreased in the lichenized form (Fig. 4B, D), this should not be interpreted as a lower capacity of the photobiont to consume or deal with excess light, since AQE remained equal to that of the free photobiont and fungal respiration can explain most of the modulation of both parameters. Indeed, the actual irradiance reaching photosynthetic cells in the lichen is very likely reduced by the melanized upper cortex and by the thicker thallus (Supplementary Data Fig. S1). If data are normalized by Chl content, however, the lichenized *Prasiola* appears much less efficient in terms of A_N (Supplementary Data Fig. S3). This could be explained by (1) the pluri-layered structure of the lichenized algae (Supplementary Data Fig. S1), related to a higher Chl content on a thallus area basis, and (2) by the fungal biomass.

Lichenized and free-living forms of *Prasiola* showed very similar photosynthetic responses to changing light conditions (Fig. 4). In agreement with this, photochemical efficiency (YII) measured in the field responded very similarly to irradiance in *P. crispa* and lichenized *Prasiola* sp. (Fig. 3A). The main difference was observed at low irradiance (e.g. PPFD <200 µmol m^{-2} s^{-1}), at which *P. crispa* showed higher YII (Fig. 3A), possibly due to a difference in its alternative sink of electron pathways, since both *P. crispa* and *M. tessellata* presented equal AQE for CO_2 fixation. At higher irradiances (>600 µmol m^{-2} s^{-1}) the two species showed similar YII (≤0.1), but much higher I_{sat90} was shown by *P. crispa* (Fig. 4D). This could be related to efficient photoprotection mechanisms. *Prasiola crispa* seems to be rather tolerant of UV (Post and Larkum, 1993; Holzinger *et al.*, 2006) and *M. tessellata* has also been shown to be very tolerant even of UV-B (Lud *et al.*, 2001*a*). Both intensity and quality of irradiance affect photo-inactivation of PSII, and while no data are available for *M. tessellata* so far, a newly identified desiccation-induced protection mechanism that prevents inactivation of the

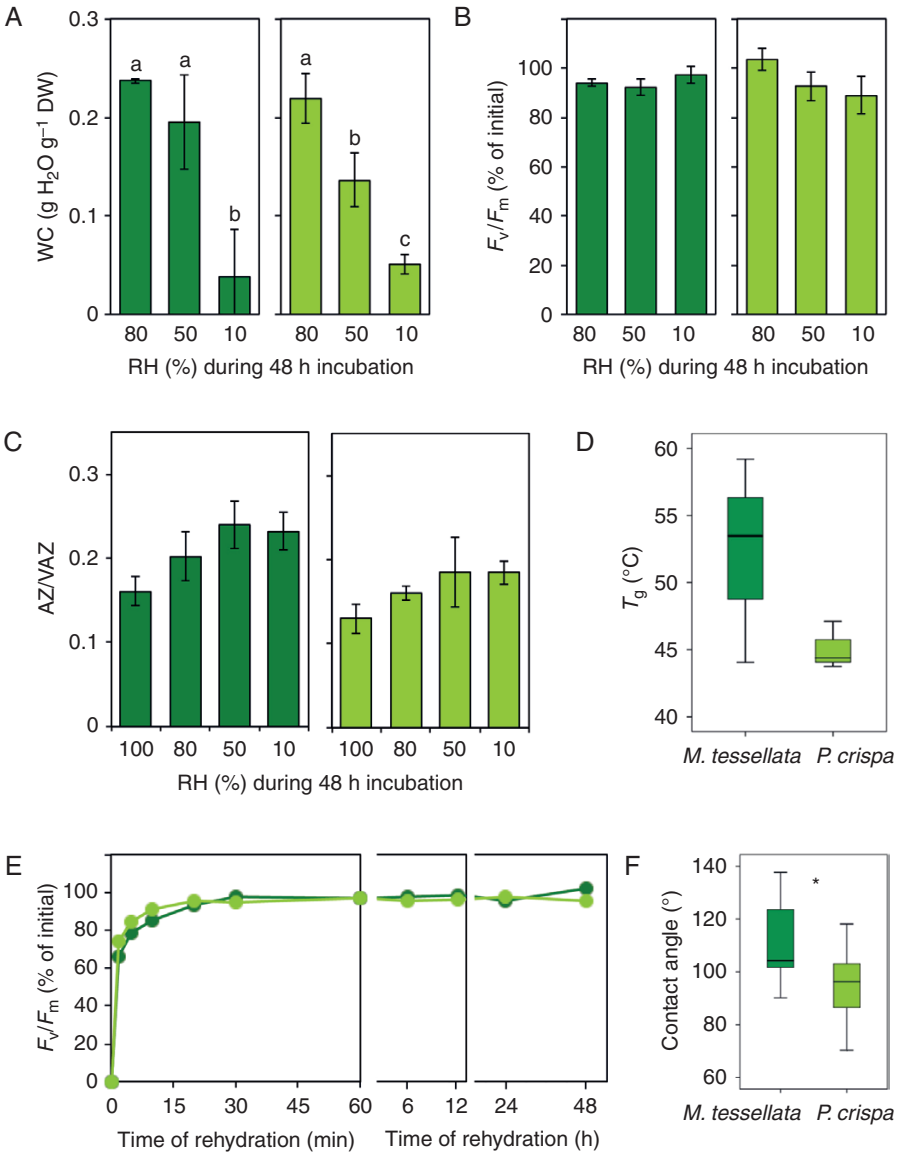

FIG. 6. Physiological, biochemical and physicochemical responses of *M. tessellata* (dark green) and *P. crispa* (light green) under controlled desiccation in darkness. (A) Final water content (WC) at the end of desiccation treatments under different relative humidities (RH). (B) Percentage of F_v/F_m recovery after desiccation and subsequent hydration. (C) De-epoxidation extent of the xanthophyll cycle pigments (AZ/VAZ) at the end of controlled desiccation. Bars are average ± s.e. ($n = 3$). (D) Glass transition temperature (T_g) of samples equilibrated at 10 % RH ($n = 3$). (E) Recovery kinetics of F_v/F_m during rehydration of samples previously equilibrated to 50 % RH. Data points are average ± s.e. ($n = 6$). (F) Static contact angle of a 3-µL distilled water drop on thallus surface ($n = 10$). When significant, differences between *P. crispa* and *M. tessellata* are depicted with an asterisk ($P < 0.05$).

oxygen-evolving complex under severely reduced water content has recently been evidenced in Antarctic *P. crispa* (Kosugi *et al.*, 2018).

Chlorophyll content per thallus dry weight was 22.6 % lower in *M. tessellata* than in *P. crispa* (2.8 ± 0.2 versus 3.6 ± 0.3 µmol g^{-1} DW, average ± s.e.; Fig. 2A). The TMA of *M. tessellata* was, on average, 5-fold that of *Prasiola*. Assuming a similar Chl content in free-living and symbiotic *Prasiola*, this would indicate that the mycobiont contributes to only one-fifth of the lichen biomass. This contribution of the mycobiont to the lichen biomass could be even lower if a reduction in the Chl content per area is assumed to be caused by the deterioration of some algal cells with massive penetration of fungal haustoria (Pérez-Ortega *et al.*, 2010). Interestingly, Chl *a*/*b*, usually considered a good indicator of light acclimation (the higher the value, the higher the light acclimation) (Esteban *et al.*, 2015), was higher in lichenized *Prasiola* (Fig. 2B). This seems counterintuitive since light-filtering by the fungal tissue should decrease light reaching the photobionts (Solhaug *et al.*, 2010), as it has been described previously that fungal hyphae form a dense network surrounding individual *Prasiola* cells (Pérez-Ortega *et al.*, 2010) and that the outermost layer of the thallus contains melanin (Lud *et al.*, 2001*b*). This fact could be explained by the preference of *M. tessellata* for more exposed microhabitat or to intrinsic differences between the free-living and symbiotic *Prasiola* species. The latter scenario could also

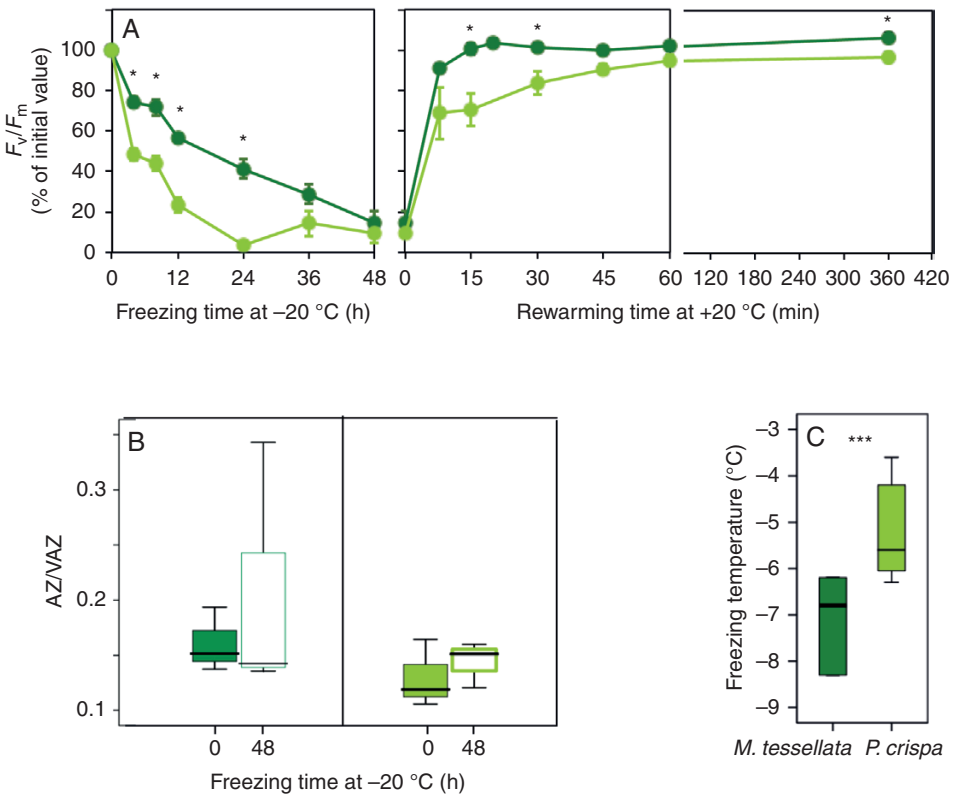

FIG. 7. Physiological and physicochemical parameters of hydrated thalli from *M. tessellata* (dark green) and *P. crispa* (light green) subjected to controlled freezing. (A) Kinetics of F_v/F_m during freezing (incubation at −20 ºC) and subsequent thawing at +20 ºC. Data points are average ± s.e. ($n = 6$). (B) De-epoxidation level of xanthophyll cycle before and after 48 h of incubation ($n = 3$). (C) Freezing temperature ranges obtained from onset temperatures of exothermic peak recorded with differential scanning calorimetry during cooling scan ($n = 6$; see Supplementary Data Fig. S5 for representative examples of the scans). When significant, differences between *P. crispa* and *M. tessellata* are depicted with an asterisk ($P < 0.05$).

explain the existence of significant differences in the ratios to Chl of some carotenoids, such as β-Car, Lut and Neo (Fig. 2). Although not evaluated in this work, the ultrastructure of the plastid is very similar in the free-living *Prasiola crispa* (Holzinger *et al.*, 2006) and the lichenized form *M. tessellata* (Pérez-Ortega *et al.*, 2010). Very few studies have dealt with the composition and dynamics of photosynthetic pigments and tocopherol in lichens generally (Demmig-Adams *et al.*, 1990; Kranner *et al.*, 2003; Fernández-Marín *et al.*, 2010; Míguez *et al.*, 2017), and these are virtually unknown at latitudes >60º except for a couple of Antarctic lichen species (Lud *et al.*, 2001*a*; Strzalka *et al.*, 2011; Sadowsky and Ott, 2012). Our data reveal higher α-Toc/Chl ratios (around 4-fold) than those found in temperate lichen species (Kranner *et al.*, 2003), which could be a species-specific trait or the combined consequence of age and stress-related factors (Lizarazo *et al.*, 2010). HPLC analyses also revealed that relatively high AZ/VAZ values are maintained continuously in the field in both species, with low re-epoxidation during nights (Figs 1D and 2F). These elevated AZ/VAZ values decreased only after several consecutive days of low irradiance, rain or both (Fig. 3B). Nevertheless, the capacity for faster re-epoxidation of xanthophyll cycle pigments was demonstrated, as samples artificially kept in darkness at high RH showed an AZ/VAZ value of 0.15 (Figs 6C and 7B), a value typical of dark-adapted non-stressed photosynthetic organisms (Verhoeven *et al.*, 1998). A similar lack of night relaxation of the xanthophyll cycle at high latitudes has been observed during summer in the case of other terrestrial photosynthetic organisms (Fernández-Marín *et al.*, 2018*a*, 2019), probably indicating an exacerbated de-epoxidation of the cycle under a long photoperiod (>12 h light).

Since lichenization has a tremendous effect in terms of carbon economy for the photobiont, the benefit of the symbiosis could be superior resistance to environmental stress. To test this hypothesis, water relations, desiccation and freezing tolerance were studied in more detail.

Water relations and tolerance of desiccation in free-living versus lichenized Prasiola

New methodological approaches are providing new perspectives on the ecophysiological interpretation and understanding of water relations in lichens (Nardini *et al.*, 2013; Bacior *et al.*, 2017; Petruzzellis *et al.*, 2018). Compared with other lichen species, *M. tessellata* showed relatively low (very negative) Ψ_{TLP} and cell wall elasticity (i.e. high ε) (Nardini *et al.*, 2013). In agreement with Petruzzellis *et al.* (2018), who compared isolated photobionts with their lichenized forms, in our study *P. crispa* samples showed higher cell wall elasticity

(e.g. lower ε), higher (less negative) Ψ_{TLP} and higher Ψ_o than *M. tessellata* (Fig. 5). Interestingly, *P. crispa* had higher SWC and lower RWC_{TLP} than the lichen, which, together with the more elastic cell wall, indicates a greater water content range for the free-living algae between saturation and TLPs. Related to this, Bacior *et al.* (2017) have recently shown similar hydration kinetics and sorption isotherms for *M. tessellata* and *P. crispa*, with the exception of a larger fraction of loosely bound water in the lichen. The presence of liquid water (rather than vapour) was previously described to be beneficial for high photosynthetic efficiency in *P. crispa* (Kosugi *et al.*, 2010). A high content of easily hydrating pectinic substances in the cell wall has already been proposed as a potential reason behind the water retention properties of *P. crispa* (Jacob *et al.*, 1992). It has been shown recently that the loss of turgor can play a key role in triggering desiccation-related responses at the photobiont cell level (Banchi *et al.*, 2018). In view of this, and based on the differences in water relations found between free-living and lichen-forming species, it would be reasonable to expect different physiological strategies against desiccation.

Our short-desiccation (48 h) approach did not support differences in desiccation tolerance between *M. tessellata* and *P. crispa* (Fig. 6). This terrestrial alga has already been described to be desiccation-tolerant (Kosugi *et al.*, 2010). The authors observed that desiccation induces a strong quenching of Chl fluorescence together with a loss of PSII activity. In agreement with the study of Kosugi *et al.*, (2010) our data showed complete inhibition of F_v/F_m in the dry state (Fig. 6E). Other species of the genus showed different extents of tolerance of desiccation. The terrestrial temperate species *P. calophylla*, for example, survives equilibration to 60 % RH for at least 2 h and without alterations in the thylakoidal structure of its chloroplasts (Holzinger *et al.*, 2017), while the arctic *P. stipitata* shows high tolerance of severe desiccation (Kang *et al.*, 2013). This same species, however, shows a deleterious effect upon long-term desiccation (e.g. 30 d) (Kang *et al.*, 2013). Long incubation in darkness also had damaging effects in *P. crispa* equilibrated at different RHs (Jacob *et al.*, 1992), and 96 h of desiccation to 5 % RH induced high mortality in an unidentified *Prasiola* incubated in the presence of light (Davey, 1989). The T_g value of *M. tessellata* and *P. crispa* in the dry state was around 10 ºC higher than previously found for silica-gel-dried mosses (Fernández-Marín *et al.*, 2013) or for resurrection angiosperms equilibrated to 50 % RH (Fernández-Marín *et al.*, 2018*b*). Although not significant, a clear trend towards higher T_g was found in *M. tessellata*, which could be related to lower molecular mobility and then slower deteriorative reactions (e.g. ageing) in the long term in the dry state (Fernández-Marín *et al.*, 2013). In the light of the determined T_g (Fig. 6D) and under the general assumption that tolerance of desiccation of lichen photobionts seems to be enhanced in their lichenized form (Kranner *et al.*, 2005; Kosugi *et al.*, 2009), *M. tessellata* may show better preservation of functional integrity in the dry state at a longer temporal scale. In agreement with this, Huiskes and co-workers found that continuous hydration promotes algal growth while desiccation promotes fungal success within the delicate equilibrium of this symbiosis (Huiskes *et al.*, 1997*a*).

In summary, our results do not support an effect of lichenization on the desiccation tolerance of *Prasiola* in the short term, although it may have a positive effect upon long-term (several days) desiccation events.

Lichenization enhances tolerance of freezing in Prasiola

The accumulation of the antioxidant and membrane stabilizer zeaxanthin upon desiccation and upon freezing conditions has been reported in different photosynthetic organisms, including resurrection plants, lichens and intertidal macroalgae (Fernández-Marín *et al.*, 2010, 2011*a*, *b*, 2013, 2018*b*; Verhoeven *et al.*, 2018). Although a trend towards higher AZ/VAZ was observed upon desiccation and at low temperature for *P. crispa* and *M. tessellata* (Figs 6C and 7B, respectively), no significant differences in this ratio were obtained under the experimental conditions used. Even though F_v/F_m decreased considerably (~90 %) after 48 h of incubation at −20 ºC in both species. It is remarkable that this decrease was slower in *M. tessellata*, and only after an incubation time longer than 24 h did the F_v/F_m drop below 50 % of the initial value (Fig. 7A). By contrast, this happened in the first 6 h of incubation in the free-living *Prasiola* (Fig. 7A). Accordingly, the ice nucleation point was significantly lower in *M. tessellata* than in *P. crispa* (Fig. 7), and 2–3 ºC lower than that reported for temperate lichen species growing at high latitudes, such as *Lobaria virens* and *Lobaria pulmonaria* (Solhaug *et al.*, 2018). The lower ice nucleation temperature in *M. tessellata* was in agreement with a slower depression in its photochemical activity along with incubation at −20 ºC and with its faster recovery upon thawing when compared with *P. crispa* (Fig. 7). While literature is available about the freezing tolerance of intertidal algae, much less is known regarding the tolerance of freezing in lichens in their hydrated state, and to the best of our knowledge virtually no study has dealt with the effects of lichenization on the enhancement of photobiont fitness under low temperature. Isolated photobionts from Antarctic lichens in the genus *Trebouxia* have been described as highly tolerant of freezing even when artificially isolated from their respective mycobionts (Sadowsky and Ott, 2012). *Prasiola crispa* seems to survive freezing (Jackson and Seppelt, 1995) and some species in the genus show the ability to photosynthesize at low temperatures, down to −15 ºC (Becker, 1982). Despite this, in the light of our results it seems that the tolerance of freezing in *Prasiola* can be enhanced in symbiosis with the fungus *M. tessellata* (Fig. 7). Two potential explanations could be argued: (1) morpho-anatomical modifications induced by the presence of fungal hyphae in the lichenized form (see Supplementary Data Fig. S1) and (2) biochemical alterations. In agreement with the former, very recent data on the water behaviour of *M. tessellata* versus *Prasiola* indicate changes in the patterns of ice crystallization induced by both the structure and the biochemistry of the lichenized forms (Bacior *et al.*, 2019). In agreement with the latter, hardening of the photobiont against environmental constraints (such as desiccation) mediated by metabolic changes (e.g. accumulation of compatible solutes, like arabitol) upon lichenization has been evidenced for some species (Kosugi *et al.*, 2013). In a similar way the contribution of cryoprotectant substances by the mycobiont could be relevant in the protection of the lichenized *Prasiola*.

Although out of the scope of this work, other potential benefits of lichenization to the photobiont, such as the reduced impact of herbivorism or pathogen infection, could be considered in future studies. Interestingly, our data also provide new evidence about how free-living algae can cope with abiotic stress without any protection by the fungal partner. This has also been observed in astrobiological experiments under extreme experimental conditions (Sánchez *et al.*, 2014) highlighting the intrinsic high stress resistance of many lichen photobionts.

Conclusions

Overall, our work provides new insights into the functioning of the symbiosis established by *Prasiola* sp. and the lichen-forming fungus *M. tessellata* in Antarctica. While lichenization appears to have no positive effect on the photochemical performance of the alga or its tolerance of desiccation (in the short term at least), the symbiosis represents (1) changes in its water relations, (2) a considerable decrease in net carbon uptake (due to fungal respiration and energy consumption) and (3) enhanced tolerance of freezing. The profound impact of lichenization on the carbon balance cannot have a neutral effect; however, there is no clear explanation of why scattered patches of both *Mastodia* and *Prasiola* coexist in the same microsites (Fig. 1). In the light of our results, enhanced tolerance of low temperature would be a relevant ecological benefit for *Prasiola* upon lichenization. All things considered, whether the overall changes in the functioning of the alga as a consequence of lichenization will be reciprocally beneficial for the symbionts or will favour the single benefit of one of the partners seems to be rather dependent on environmental factors. It has already been reported that warmer winters could be deleterious to freezing-tolerant lichens (Bjerke, 2011) and that the differences in A_n between *P. crispa* and *M. tessellata* are exacerbated under long-term incubation in the hydrated state (Huiskes *et al.*, 1997*b*). In a climate change scenario, a rise in temperature that would (1) led to depressed A_n and (2) reduce the benefits of frost tolerance when lichenized would very likely lead to the spread of the free-living alga to the detriment of the lichen form, with resulting alterations of unknown consequences in Antarctic ecosystems.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following.

Figure S1: details of *M. tessellata* and *P. crispa* anatomy obtained by optical microscopy.

Figure S2: gas exchange data of both species on a DW basis.

Figure S3: gas exchange data of both species on a chorlophyll content basis.

Figure S4: carbon assimilation curve of *P. crispa* and *M. tessellata* measured at +10 ºC with a CMS 400 photosynthesis system.

Figure S5: DSC scans of hydrated thalli from *M. tessellata* and *P. crispa* for estimation of freezing temperature.

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