Drought and frost resistance vary between evergreen and deciduous Atlantic Forest canopy trees

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Abstract. Frost and drought are key stress factors limiting the growth and distribution of tree species. Resistance to stress involves energy costs that may result in trade-offs between different functional traits. Structures or mechanisms that can help to withstand stress imply differences in the carbon economy of the species. Although adaptive responses to frost and drought resistance are usually of a similar nature, they are rarely assessed simultaneously. We investigated these resistance mechanisms in 10 canopy tree species coexisting in the semi-deciduous subtropical forests of northern Argentina. We measured leaf lifespan, anatomical, photosynthetic and water relations traits and performed a thermal analysis in leaves to determined ice nucleation and tissue damage temperatures. Our results showed that evergreen and deciduous species have different adaptive responses to cope with freezing temperatures and water deficits. Evergreen species exhibited cold tolerance, while deciduous species were more resistant to hydraulic dysfunction and showed greater water transport efficiency. Further research is needed to elucidate resistance strategies to stress factors at the whole tree- and stand level, and possible links with hydraulic safety and efficiency among different phenological groups. This will allow us to predict the responses of subtropical forest species to changes in environmental conditions under climate change scenarios.

Additional keywords: electron transport rate, frost resistance, hydraulic traits, leaf economics spectrum, leaf habit, trade-off, turgor loss point, water availability.

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Introduction

Resistance of species to stress events is determined by the interaction of multiple functional traits (Bartlett *et al.* 2016). The interspecific variation of these traits can increase species richness (Ben-Hur *et al.* 2012) and promote their coexistence (Clark *et al.* 2010; Uriarte *et al.* 2010; Anderegg and Hillerislambers 2016). Low temperatures and water availability are two of the main stressors limiting the growth and geographical distribution of tree species growing beyond the tropical regions. Both stress factors affect important plant physiological processes such as photosynthesis, photoinhibition, and transpiration (Sakai and Larcher 1987; Stuart *et al.* 2007; Charra-Vaskou *et al.* 2012).

The exchange of gases through stomata, and the fixation of carbon in photosynthetic tissues are two important physiological processes in leaves (Poorter *et al.* 2008; Martínez-Vilalta *et al.* 2010; Wright *et al.* 2010; Iida *et al.* 2016). To operate optimally, these processes require a well-balanced hydration status of the leaf. This water status is associated with stomatal regulation as well as water transport in leaves through inside and outside the xylem pathway (Prado and Maurel 2013; Buckley *et al.* 2015; Scoffoni *et al.* 2017). Small variations in the water flow through leaves have important implications on the water transport at plant level (Holbrook and Zwieniecki 2005), and could strongly limit productivity, competitive

success, and ultimately species distribution (Sack and Holbrook 2006).

Evergreen and deciduous species represent the opposite extremes in the leaf economics spectrum from conservative and slow-growing to acquisitive and fast-growing, respectively, as suggested by correlations among photosynthetic rate, leaf lifespan (LL), and specific leaf area (SLA) (Wright *et al.* 2004; Bai *et al.* 2015). Further, the leaf economics framework was extended to a more general plant economic spectrum incorporating wood and hydraulic traits (Reich 2014; Xu *et al.* 2017). Wood density (WD) is functionally related to carbon gain, and regulation of leaf water status (Santiago *et al.* 2004; Jacobsen *et al.* 2007; Chave *et al.* 2009; Oliva Carrasco *et al.* 2015).

Adaptations to freezing and drought often share similar physiological mechanisms (Atkin *et al.* 2005; Beck *et al.* 2007; Costa e Silva *et al.* 2009). Tree species exhibit three strategies to resist these stress factors: escape, avoidance, and tolerance (Levitt 1980; Hódar *et al.* 2004; Körner 2012). Deciduous trees usually escape the effect of freezing or drought by adjusting leaf phenology to seasonal changes (Cavender-Bares *et al.* 2005; Pennington *et al.* 2009; Hasselquist *et al.* 2010; Wolfe and Kursar 2015). Avoidance can be regarded as the minimisation or the exclusion of the stress effect on a given tissue (Vitasse *et al.* 2014). Examples of this include supercooling (Goldstein *et al.* 1985; Wisniewski *et al.* 2015), rolling of leaves (Schwabe and Lionakis 1996) and strongly lignified tissues (Richardson and Berlyn 2002). Tolerance is achieved via morphological, anatomical, and biochemical changes to allow osmotic and elastic adjustment of tissues (Preston and Sandve 2013). Both drought- and freezing-tolerant species resist cell dehydration (Charra-Vaskou *et al.* 2012). Although frost and drought resistances in leaves may be correlated (Medeiros and Pockman 2011), simultaneous measurements and analysis on these adaptive responses for a large number of species are lacking.

The Atlantic Forest biome is located in South America, covering a great extension of the Brazilian coast and extending from up to 700 km to the continent to Argentina and Paraguay. In its subtropical part, deciduous trees comprise a substantial percentage (25–50%) of the species (Leite and Klein 1990; Rezende *et al.* 2016). Despite the mild seasonality in temperatures and rainfall, there is little research examining plant strategies in subtropical forests and, particularly, in the semi-deciduous Atlantic Forest. The aim of the present study was to evaluate the resistance mechanisms to drought and low temperatures in relation to leaf habit, wood density, and leaf anatomical, hydraulic, and photosynthetic traits in 10 canopy tree species coexisting in the semi-deciduous Atlantic Forest in NE Argentina. Mechanisms of stress resistance imply trade-offs among different functional traits with high energy costs (Xu *et al.* 2009; Onoda *et al.* 2011). According to the leaf economic spectrum framework we expected that species with long leaf lifespan have traits that allow them to avoid or tolerate frost and drought stress. Species with short leaf lifespan, which escape subzero temperatures, have leaf traits associated with maximising carbon assimilation

and water transport efficiency during the growing season at the expense of drought resistance.

Materials and methods

Study site

The present study was conducted in the semi-deciduous Atlantic Forest at Iguazú National Park, Misiones province, Argentina (25°41′–25°42′S, 54°28′–54°29′W). This forest has mature canopy trees ranging from 20 to 45 m in height, with abundant lianas and epiphytic species (Srur *et al.* 2007). This subtropical forest contains more than 70 tree species (Galindo-Leal and Gusmão Câmara 2003) including evergreen, and winter deciduous and brevideciduous species (Pennington *et al.* 2009). The mean annual rainfall is 2000 mm, evenly distributed throughout the year. Exceptionally intense and short-lived droughts may occur during the year, where soil water potentials can attain values as low as −2MPa (Campanello *et al.* 2009). The annual mean air temperature is 21°C. Additionally, freezing temperatures (frosts) of 2–9 days per year occur during autumn–winter (Srur *et al.* 2007; Gatti *et al.* 2008).

In order to evaluate the traits associated with resistance to low temperatures and water deficits, thermal analyses and measurements of hydraulic, anatomical, and photosynthetic leaf traits were carried out in 10 tree canopy species of the semi-deciduous Atlantic Forest: *Ceiba speciosa* (A.St.-Hil., A. Juss. & Cambess.) Ravenna, *Cedrela fissilis* Vell., *Cordia trichotoma* (Vell.) Arráb. Ex Steud., *Cabralea canjerana* (Vell.) Mart., *Ocotea diospyrifolia* (Meisn.) Mez., *Chrysophyllum gonocarpum* (Mart. & Eichler) Engl, *Balfourodendron riedelianum* (Engl.) Engl., *Lonchocarpus muehlbergianus* Hassl, *Parapiptadenia rigida* (Benth.) Brenan, and *Holocalyx balansae* Micheli. Values of WD employed in this study were obtained according to Oliva Carrasco *et al.* (2015). Species selection was based according to their relatively high abundance, which additionally covered a wide range of WD values, and represented different leaf habits characteristic of the semi-deciduous Atlantic Forest (Zuloaga *et al.* 2008; Jardim Botânico do Rio de Janeiro 2018; see di Francescantonio *et al.* 2018 for details).

Monthly precipitation and temperature data for the study period (see Fig. S1, available as Supplementary Material to this paper) were obtained from the nearest (8 km) meteorological station (Puerto Iguazú-Aero, Servicio Meteorológico Nacional, Argentina, 25°43′48″S–54°28′12″W).

Leaf lifespan

In 2012, two trees per species were selected to measure LL. Depending on the species, two to three branches between 13 and 18 m high were selected, where a minimum of 20 recently expanded leaves with unique identifiers was marked. The presence of tagged leaves was monthly recorded. Survival time and LL for each species at the time from full leaf expansion to abscission were estimated with the Kaplan–Meier method (Kitajima *et al.* 2013; Russo and Kitajima 2016), which accounts for censored leaves (leaves

removed from the study before their death or alive at the last survival census).

Anatomical and photosynthetic leaf traits

The methodologies described in Pérez-Harguindeguy *et al.* (2013) were used for determining specific leaf area (SLA) and venation traits in 10 and three leaves per species, respectively. The leaves were cleared to quantify venations traits. For this purpose, leaves were boiled in 96% alcohol, and then in a solution of 5% NaOH and 96% ethanol, 1 : 1 (v/v). The time of boiling varied according to the hardness of leaves tissue. Then, leaves were washed in distilled water and transferred into a sodium hypochlorite solution, until they were transparent. Finally, the tissues rich in lignin were stained with a solution of 2% safranin in ethanol, and scanned at 600 dpi. Whole cleared leaf images were measured to estimate leaf area, and density of 1st and 2nd veins. Vein density (VD) was measured as the total length of the 1st and the 2nd order leaf venations per leaf area using ImageJ (NIH Image) by manually drawing and counting all veins in the leaf area. The leaves used to study venation were also used to determine the stomatal density (SD). Clear nail polish was applied onto the middle of the abaxial and adaxial leaf surface (avoiding the major vein). After they were dried, they were peeled off and mounted onto a glass slide to obtain images that were captured using a light microscope with $40\times$ magnifications (Leica Lietz DMRB; Leica Microsystems, with Nikon Coolpix 4500 digital) (Russo *et al.* 2010).

To estimate leaf photosynthetic capacity a chlorophyll fluorescence analyser with a pulse-amplitude modulated yield (Mini-PAM Walz) was used. Three to four individuals per species were selected during spring. Leaves used had similar size and sun exposure. Electron transport rate (ETR) was calculated based on chlorophyll fluorescence data using the following equation:

$$ETR = \Phi PSII \times PFD \times 0.5 \times \alpha,$$

where ETR is electron transport rate, PSII is the effective quantum yield of $\Phi PSII$ measured during a 0.8-s saturating flash (2000–3000 μmol m^{-2} s^{-1}), and the coefficient of 0.5 factor assumes an even distribution of absorbed quanta between PSII and PSI so that two photons are required for each electron passed through (Maxwell and Johnson 2000). The α parameter was assumed to be 0.84 as a mean value for a wide variety of leaves (Björkman and Demmig 1987; Stemke and Santiago 2011). Photosynthetic capacity, expressed as the maximum electron transport rate (ETR$_{max}$; μmol m^2 s^{-1}), was calculated from the light curves as described by Rascher *et al.* (2000):

$$y = (ETR_{max})(1 - e^{-bx}),$$

where y is photosynthetic rate, x is irradiance, and b is the instantaneous fractional growth rate of the exponential function.

Thermal analysis and tissue damage

Ice nucleation temperatures of leaves

Thermal analyses of mature leaves were conducted to determine the ice nucleation temperature (INT). During the months of April and May 2016, sun-exposed branches from three individuals of each species were collected early in the morning and immediately covered by black plastic bags and transported to the laboratory. Leaves were placed in a temperature controlled freezer in contact with copper-constantan thermocouples in order to determine tissue temperature. One thermocouple was attached with surgical tape (Transpore, 3M) against the underside of each leaf and was positioned in a tube within the freezer. The thermocouples were connected to an automatic data acquisition system (CR10X, Campbell Scientific), and temperatures were recorded at 4-s intervals. Freezer temperature was lowered at a rate of 5°C h^{-1} from ambient (20°C) to –10°C. This rate was similar to those used in other studies (e.g. Lipp *et al.* 1994; Arias *et al.* 2015). Foliar INT was obtained from the tissue temperature kinetics. A rapid increase in temperature indicated heat release from water during extracellular ice nucleation.

Leaf tissue damage

The electrolyte leakage method was used to assess the impact of low temperatures on leaf tissue damage (Wilner 1960; Lipp *et al.* 1994). Leaves samples from the same branches used in thermal analysis were placed into sealed tubes (16 leaf discs per tube, 11 tubes per individual, three individuals per species) and incubated in a freezer. The freezer was then cooled down at a rate of 5°C h^{-1} to reach different target temperatures (20, 10, 7, 5, 2, 0, –2, –4, –6, –8 and –10°C). After maintaining the samples at the target temperature for 15 min (Lipp *et al.* 1994), 3 tubes per species (one of each individual) were removed from the freezer and thawed at ambient-temperature (20°C) for 2 h, and then 20 mL of deionised water was added to each tube. The tubes were held at 20°C for 24 h with occasional mixing and shaking, and then the electrical conductivity (EC) of the solution was measured with an electrical conductance/resistance meter (Lutron WA-2015). The samples were frozen at –20°C for 24 h to achieve a complete electrolyte leakage, and then the EC of the solution was measured once again. The relative EC was calculated as a percentage:

$$relative\ EC = (EC\ after\ the\ temperature\ treatment/EC$$
$$at - 20°C) \times 100.$$

The % EC was plotted as a function of the incubation temperature. The temperature at 50% relative EC was defined as the leaf lethal temperature of the species (LT$_{50}$) which could be associated with membrane structural transitions (Rajashekar *et al.* 1979).

The thermal safety margin (TSM) was defined for each species as:

$$TSM = INT - LT_{50}.$$

Species that exhibited LT$_{50}$ within the range of INT (TSM = 0) were categorised as freezing-avoidant species (FAS), in cases where LT$_{50}$ was greater than INT (TSM <0) the species were considered sensitive to extracellular ice formation (freezing sensitive species, FSS), whereas species with LT$_{50}$ being sustainably lower than INT (TSM >0) were categorised as freezing-tolerant species (FTS) (Squeo *et al.* 1991; Zhang *et al.* 2016).

Leaf water relations

Leaf water potentials at predawn (Ψ_{pd}) and midday (Ψ_{md}) were measured in 4–5 leaves in three individuals per species with a pressure chamber (PMS1000) during a 30-day dry spell on January 2012. The Ψ_{md} values obtained in these conditions reflect the minimum leaf water potential for these species under natural conditions (Bhaskar and Ackerly 2006). The Ψ_{pd} and Ψ_{md} were determined at 05:30–07:00 hours and 12:30–14:00 hours respectively.

Pressure–volume analyses were obtained using the dehydration technique (Tyree and Hammel 1972; Koide *et al.* 1989). Branches of three individuals per species were collected at predawn and transferred immediately to the laboratory in sealed plastic bags. Volumetric bulk modulus of elasticity (ε) was calculated over the full range of positive turgor as described by (Evans *et al.* 1990):

$$\varepsilon = (\Delta\Phi P / \Delta RWC),$$

where $\Delta\Psi P$ is the change in turgor pressure and ΔRWC is the change in relative water content and FS is the symplastic water fraction.

Leaf hydraulic conductance (K_{leaf}; mmol m^{-2} s^{-1} MPa^{-1}) was determined according to the partial rehydration method (Brodribb and Holbrook 2003). The K_{leaf} was calculated as:

$$K_{leaf} = C\ln(\Psi_i\Psi_f)/t,$$

where C is leaf capacitance, Ψ_i is the foliar water potential before rehydration and Ψ_f is the foliar water potential after rehydration for t seconds. Foliar capacitance was determined from the P–V relationships. Leaf vulnerability was determined by plotting K_{leaf} against Ψ_i. The water potential at which loss of 50 and 88% of leaf hydraulic conductance occurs (P_{50} and P_{88} respectively) and K_{leaf_max} was estimated from a four- parameter logistic function fitted to the data.

The hydraulic safety margin (HSM), which reflects the preservation of hydraulic integrity, was calculated as follows:

$$HSM_{P50} = \Psi_{md} - P_{50},$$

$$HSM_{P88} = \Psi_{md} - P_{88},$$

where Ψ_{md} is minimum leaf water potential, P_{50} and P_{88} are the leaf water potential inducing a 50 and 88% loss in the leaf hydraulic conductance respectively.

Statistical analysis

Sigmoid curves were fitted to the relationship between relative EC and temperature for all the species. Then, LT_{50} were derived from the regression equations. P_{50}, P_{88} and K_{leaf_max} were estimated from a four-parameter sigmoid function fitted to the data. The relationship between INT, and LT_{50} with leaf habits (deciduous, brevideciduous and evergreen) were analysed using two independent linear mixed-effects models. Leaf habit was considered as a fixed effect and species a random effect. A model with a normal distribution and an identity link function was fitted to the data using the lme function (package nlme) (Pinheiro *et al.* 2017) in the R statistical and programming environment (R Development Core Team 2016). The significance of fixed effects was assessed using Fisher's test and post-hoc comparisons were carried out using Tukey's test in the multcomp R package (Hothorn *et al.* 2016). Finally, to evaluate trait coordination between deciduous-brevideciduous and evergreen species, statistically significant correlations among traits (with r >0.6, and $P < 0.05$) were graphically represented using trait covariation networks. Traits were represented as nodes and their correlation as the edges linking them. Two indicators of network centrality were calculated for each trait: the degree (D), defined as the number of edges of a node and the weighted degree (Dw), defined as the sum of all significant coefficients of correlation of a node. Pearson correlations and Kaplan–Meier method were performed with InfoStat software (Di Rienzo *et al.* 2017).

Results

Leaf lifespan

Evergreen species, with leaves always present, showed LL from 438 to 479 days; brevideciduous species, with leaves absent for 1 month or less of the year, presented LL of 269–354 days; and deciduous species, with leaves absent for more than 1 month, presented LL of 208 and 329 days (Table 1; Fig. S2).

Anatomical traits and specific leaf area

No relationship was found between leaf habits and stomatal density ($F_{2,27} = 0.63$, $P = 0.54$), or density of major veins ($F_{2,27} = 1.58$, $P = 0.22$). There were considerable differences

**Table 1.   Family, species, leaf habit, leaf anatomical traits and wood density**

Stomatal density (SD, stomatal mm^{-2}), vein density, (VD, mm mm^{-2}), maximum leaf lifespan (LL$_{max}$, days), specific leaf area (SLA, cm^2 g^{-1}), wood density (WD, g cm^{-3}). D, deciduous; B, brevideciduous; E, evergreen. Values are means $\pm$ s.e. ($n = 3$ for SD and VD; $n = 20$ for LL$_{max}$; $n = 6$–10 for SLA and WD)

Family	Species	Leaf habit	SD	VD	LL$_{max}$	SLA	WD
Meliaceae	*Cedrela fissilis*	D	142.8 ± 3.0	2.3 ± 0.4	329	97.6 ± 9.2	0.49 ± 0.01
Boraginaceae	*Cordia trichotoma*	D	33.6 ± 5.2	1.2 ± 0.02	208	66.7 ± 5.3	0.59 ± 0.02
Malvaceae	*Ceiba speciosa*	D	36.2 ± 1.0	3.2 ± 0.3	210	105.2 ± 7.4	0.41 ± 0.02
Rutaceae	*Balfourodendron riedelianum*	B	38.8 ± 2.0	2.2 ± 0.1	319	115.1 ± 12.7	0.66 ± 0.02
Fabaceae	*Parapiptadenia rigida*	B	90.2 ± 7.5	12.6 ± 1.3	269	114.5 ± 11.8	0.78 ± 0.02
Fabaceae	*Lonchocarpus muehlbergianus*	B	-	2.1 ± 0.1	354	143.1 ± 12.3	0.76 ± 0.03
Meliaceae	*Cabralea canjerana*	E	23.0 ± 3.0	1.6 ± 0.1	438	116.7 ± 14.0	0.56 ± 0.02
Lauraceae	*Ocotea diospyrifolia*	E	39.4 ± 2.4	1.6 ± 0.1	442	112.7 ± 2.5	0.56 ± 0.01
Fabaceae	*Holocalyx balansae*	E	61.2 ± 0.5	11.9 ± 0.1	479	113.9 ± 7.6	0.83 ± 0.01
Sapotaceae	*Chrysophyllum gonocarpum*	E	85.9 ± 7.5	2.4 ± 0.5	468	118.1 ± 26.4	0.66 ± 0.02

among species. Stomatal density ranged between 23.0 ± 3.0 stomata mm^{-2} in *C. canjerana* to 142.8 ± 3.0 stomata mm^{-2} in *C. fissilis*; whereas major vein density ranged between 1.2 ± 0.02 mm mm^{-2} in *C. trichotoma* to 12.6 ± 1.3 mm mm^{-2} in *P. rigida* (Table 1). Species with trichomes on the leaf surface (*C. fissilis* and *C. trichotoma*) showed the lower values in SLA (Table 1).

Thermal analysis and tissue damage

The relationship between membrane damage (EC), and the leaf tissue temperature was described by a sigmoid function for all species (Fig. S3). INT as well as the temperature at which 50%
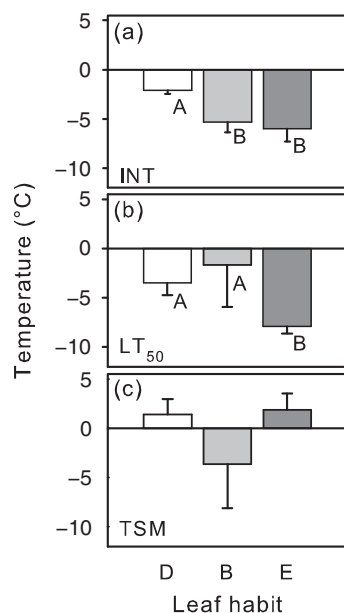
Fig. 1. Ice nucleation temperature (*a*), leaf lethal temperature (*b*), and thermal safety margin (*c*) for leaf habits. D, deciduous species (*Cedrela fissilis, Cordia trichotoma, Ceiba speciosa*); B, brevideciduous species (*Balfourodendron riedelianum, Parapiptadenia rigida, Lonchocarpus muelhlbergianus*); E, evergreen species (*Cabralea canjerana, Ocotea diospyrifolia, Holocalyx balansae, Chrysophyllum gonocarpum*). Different letters indicate significant differences at $P < 0.05$.

membrane electrolyte leakage occurred (i.e. leaf lethal temperature, LT_{50}) varied across species and was different between leaf habits ($F_{2,27} = 12.10$, $P < 0.001$; $F_{2,27} = 7.05$, $P < 0.01$ respectively; Fig. 1*a, b*; Table 2), whereas TSM did not show significant differences ($F_{2,27} = 2.86$, $P = 0.07$; Fig. 1*c*). Deciduous species had higher nucleation temperatures and LT_{50} than the evergreen species. FTS was observed in the evergreen species and in one of the brevideciduous species studied, with LT_{50} substantially lower than the INT values. FAS was observed in the deciduous and in one brevideciduous species. In these cases, LT_{50} was within the range of the INT (Table 2). Two species (*L. muehlbergianus* and *H. balansae*) were FSS, their LT_{50} was greater than INT.

The INT and LT_{50} were negatively related to LL_{max} ($r = -0.51$, $P = 0.03$ and $r = -0.77$, $P < 0.001$ respectively). Species with longer LL_{max} showed lower values of INT and LT_{50} (Fig. 2*a, b*; Table 2). WD showed a significant negative relationship with INT ($r = -0.67$, $P = 0.001$), but there was no relationship between WD and LT_{50} ($r = -0.27$, $P = 0.17$) (Fig. 2*c, d*).

Leaf water relations and photosynthetic leaf traits

The isohydricity (daily $\Delta\Psi$) of the 10 species studied showed a continuum of responses, not depending on their leaf habit (Fig. S4; Table 3). The K_{leaf} decreased as Ψ_{leaf} became more negative (Fig. S5). In general, K_{leaf_max} was higher in deciduous than in evergreens species (Table 3). Also, K_{leaf_max} varied widely among species, being six times greater in *C. speciosa* than in *C. gonocarpum* (Table 3). Ψ_{md} did not vary across the different leaf habits ($F_{2,27} = 0.59$, $P = 0.56$; Fig. 3*a*), whereas significant differences were found in P_{50} and P_{88} ($F_{2,27} = 21.7$, $P < 0.001$; $F_{2,27} = 12.7$, $P < 0.001$, respectively, Fig. 3*b, c*). Deciduous species lost hydraulic conductivity at more negative water potentials than evergreens. The HSM_{P88} also varied with leaf habit ($F_{2,27} = 19.55$, $P < 0.001$). These safety margins were positive or near zero in deciduous and brevideciduous species and negative in evergreen species (Fig. 3*d*).

The ETR_{max} was negatively related to LL ($r = -0.6$, $P = 0.03$), while no relationship was found with wood

Table 2. **Extracellular ice nucleation temperature (INT, °C), leaf lethal temperature (LT_{50}; the temperature at which 50% membrane leakage electrolyte occurred, °C), thermal safety margin (TSM, °C) and frost resistance adaptations**
Abbreviations: FAS, freezing avoidant species; FTS, freezing tolerant species; FSS, freezing sensitive species. Values are means $\pm$ s.e. ($n = 3$). Significant differences are indicated: *, $P < 0.05$; **, $P < 0.001$

Species	INT	LT_{50}	TSM	Frost resistance
Cedrela fissilis	-1.45 ± 0.13	-5.41 ± 1.20	3.96 ± 2.28	FAS
Cordia trichotoma	-2.27 ± 0.38	-3.88 ± 1.57	1.61 ± 0.85	FAS
Ceiba speciosa	-2.60 ± 0.62	-1.21 ± 0.76	-1.39 ± 1.25	FAS
Balfourodendron riedelianum	-3.53 ± 0.52	-6.24 ± 0.12	2.71 ± 0.45	FTS*
Parapiptadenia rigida	-7.01 ± 0.93	-5.62 ± 0.40	-1.39 ± 0.79	FAS
Lonchocarpus muehlbergianus	-5.50 ± 0.28	6.79 ± 1.38	-12.30 ± 1.10	FSS**
Cabralea canjerana	-3.78 ± 0.22	-7.29 ± 1.04	3.51 ± 0.85	FTS*
Ocotea diospyrifolia	-6.92 ± 0.12	-9.68 ± 0.01	2.76 ± 0.11	FTS**
Hlocalyx balansae	-9.27 ± 0.68	-6.14 ± 0.12	-3.13 ± 0.59	FSS*
Chrysophyllum gonocarpum	-4.12 ± 0.38	-8.42 ± 0.69	4.30 ± 0.71	FTS*
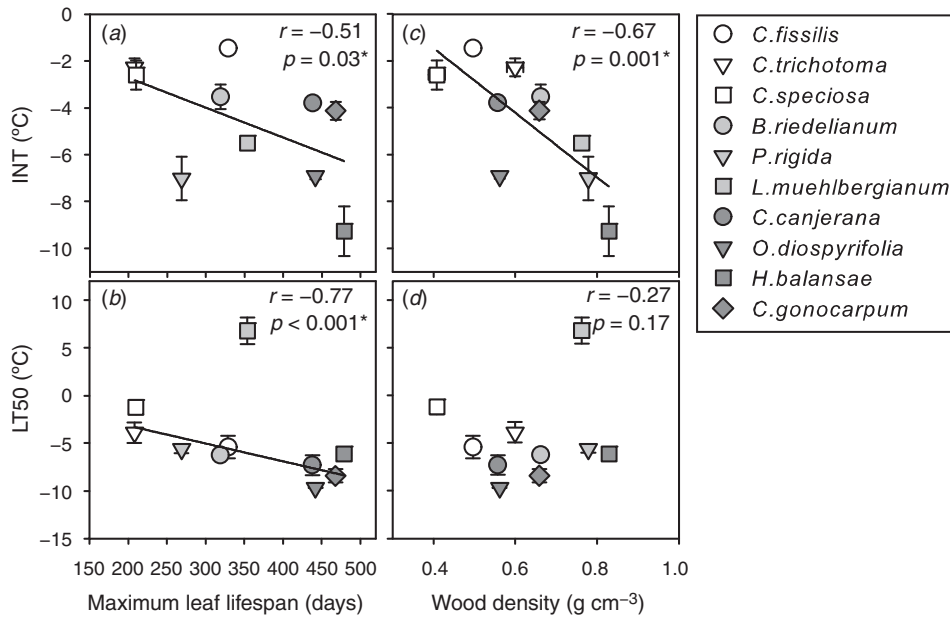
Fig. 2. Relationship between ice nucleation temperature (INT), and lethal leaf temperature (LT50) with the maximum leaf lifespan (*a, b*), and with wood density (*c, d*). Each point represents a species average and error bars represent ± s.e. *Lonchocarpus muehlbergianum* was excluded from the correlation in (*b*) because this species has the capacity of rolling leaves in responding to environmental stressors. The methodology used to evaluate leaf tissue damage does not allow the leaves to curl up and prevent damage from low temperatures.

Table 3.   Leaf hydraulics traits

Abbreviations: TLP, turgor loss point (MPa); Ψ_{md}, leaf water potential at midday (MPa); $\Psi_{pre-dawn} - \Psi_{md}$, daily $\Delta\Psi$; K_{leaf_max}, maximum leaf hydraulic conductance (mmol m^{-2} s^{-1} MPa^{-1}), P$_{50}$, hydraulic safety margin at P$_{50}$ (HSMP$_{50}$, MPa); P$_{88}$, hydraulic safety margin at P$_{88}$ (HSMP$_{88}$, MPa)

Species	TLP	Ψ_{md}	$\Delta\Psi$	K_{leaf_max}	P$_{50}$	P$_{88}$	HSM$_{P50}$	HSM$_{P88}$
Cedrela fissilis	-1.18 ± 0.02	-1.45 ± 0.09	1.12 ± 0.09	35.64 ± 3.4	-1.13	-2.46	-0.32	1.01
Cordia trichotoma	-2.48 ± 0.32	-2.65 ± 0.39	2.22 ± 0.32	45.00 ± 2.8	-0.86	-1.55	-1.79	-1.1
Ceiba speciosa	-1.6 ± 0.05	-1.10 ± 0.03	0.92 ± 0.1	48.46 ± 4.6	-0.66	-1.52	-0.44	0.42
Balfourodendron riedelianum	-2.12 ± 0.04	-2.42 ± 0.24	1.53 ± 0.33	13.4 ± 1.2	-2.4	-3.43	-0.02	1.01
Parapiptadenia rigida	-2.24 ± 0.07	-2.68 ± 0.09	1.4 ± 0.05	8.86 ± 0.9	-1.86	-3	-0.82	0.32
Lonchocarpus muehlbergianus	-1.68 ± 0.04	-0.4 ± 0.03	0.15 ± 0.03	39.14 ± 3.2	-0.42	-0.55	0.02	0.11
Cabralea canjerana	-1.42 ± 0.09	-1.45 ± 0.55	1.55 ± 0.56	27.06 ± 4.1	-0.49	-1.55	-0.96	0.1
Ocotea diospyrifolia	-2.69 ± 0.13	-2.36 ± 0.09	2.03 ± 0.14	8.34 ± 0.8	-0.52	-0.75	-1.84	-1.61
Holocalyx balansae	-1.87 ± 0.11	-2.54 ± 0.08	1.43 ± 0.23	18.49 ± 1.3	-0.38	-0.9	-2.16	-1.64
Chrysophyllum gonocarpum	-2.47 ± 0.29	-2.25 ± 0.03	2.02 ± 0.03	7.51 ± 0.8	-0.56	-0.98	-1.71	-1.29

density ($r = -0.3$, $P = 0.08$) (Fig. 4*a, d*). Evergreen species, with longer LL, showed lower ETR$_{max}$, whereas deciduous-brevideciduous species (with lower LL) the higher ETR$_{max}$ values. The K_{leaf_max} was negatively related to LL ($r = -0.6$, $P = 0.002$), and with WD ($r = -0.5$, $P = 0.01$). Species with longer LL and higher WD showed the lower values of K$_{leaf\_max}$ (Fig. 4*b, e*). The bulk elastic modulus (ε) was positively related with LL ($r = 0.5$, $P = 0.01$), but not with WD ($r = 0.3$, $P = 0.07$, Fig. 4*f*). Species with higher ε (higher cell wall rigidity) had a longer LL (Fig. 4*c*).

Leaf traits correlations network

Trait coordination was different between deciduous-brevideciduous and evergreen species (Fig. 5; Table S1).

Evergreens showed a more complex correlation network, with more and different connections than deciduous-brevideciduous species (Table S2). In deciduous-brevideciduous species, P$_{50}$, P$_{88}$, and K_{leaf_max} were the traits showing the highest values of centrality (Fig. 5*a*; Table S2). K$_{leaf\_max}$, P$_{50}$, and P$_{88}$ were positively related, indicating that species with higher water transport efficiency were more vulnerable to hydraulic dysfunction. These three traits were positively related to LT$_{50}$, higher $K_{leaf\text{-}max}$ was associated with lower resistance to low-temperature damage (LT$_{50}$), whereas to lower cavitation resistance (i.e. more positive P$_{50}$ and P$_{88}$) implies lower resistance to low-temperature damage (LT$_{50}$). In addition, P$_{50}$, P$_{88}$, and K_{leaf_max} were negatively related to leaf tissue elasticity (ε),
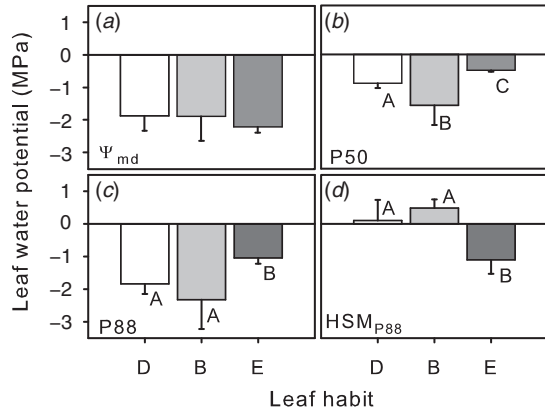
Fig. 3. (*a*) Leaf water potential at midday (Ψ_{md}), (*b*) water potential at 50% (P_{50}), and (*c*) 88% (P_{88}) loss of leaf hydraulic conductance, and (*d*) safety margin hydraulic safety margin (HSM)$_{P88}$ for leaf habits. D, deciduous species; B, brevideciduous species; E, evergreen species. Different letters indicate significant differences between leaf habits at $P < 0.05$.

so more elastic leaf tissue (lower ε) were related to lower cavitation resistance (more positive P_{50} and P_{88}), and lower water transport efficiency (Fig. 5*a*).

In traits correlation network of evergreens species, ε and turgor loss point (TLP) were the traits showing the highest values of centrality (Fig. 5*b*; Table S2). The ε and TLP were negatively related. The ε was negatively associated with LT_{50}, ETR_{max}, HSM_{P88} and K_{leaf_max} (i.e. plants with more rigid leaf tissue have also lower resistance to cold damage, lower maximum electron transport rate, hydraulic safety margin, and water transport efficiency), and positively to P_{88} (more rigid leaf tissue implies a larger vulnerability to hydraulic dysfunction). The TLP was negatively related to P_{88} and positively related with LT_{50}, ETR_{max}, HSM_{P88} and K_{leaf_max}. Evergreens showed negative relationships between P_{50}, P_{88} and INT, and a positive relationship with LT_{50}, so higher vulnerability to hydraulic dysfunction imply higher temperatures of ice nucleation but also higher resistance to low-temperature damage. Also, evergreens showed a negative correlation between INT and P_{50} and a positive correlation
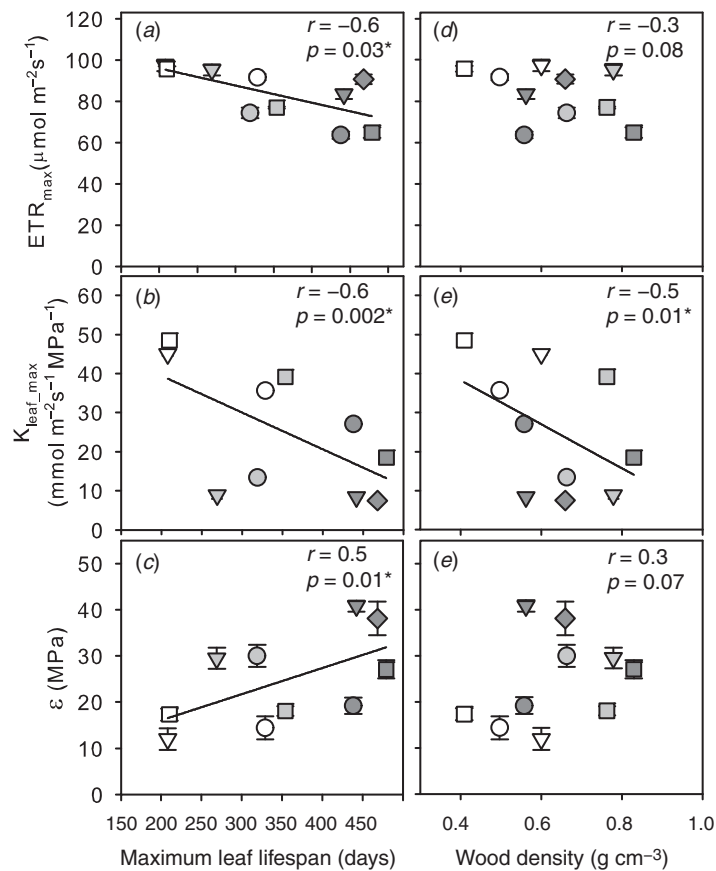
Fig. 4. Relationship between maximum electron transport rate (ETR_{max}), leaf hydraulic conductance (K_{leaf_max}), and volumetric bulk modulus of elasticity (ε), with the maximum leaf lifespan (*a–c*), and with wood density (*d–f*). White symbols, deciduous species; light grey symbols, brevideciduous species; dark grey symbols, evergreen species. Different symbol shapes represent different species. See Fig. 2.
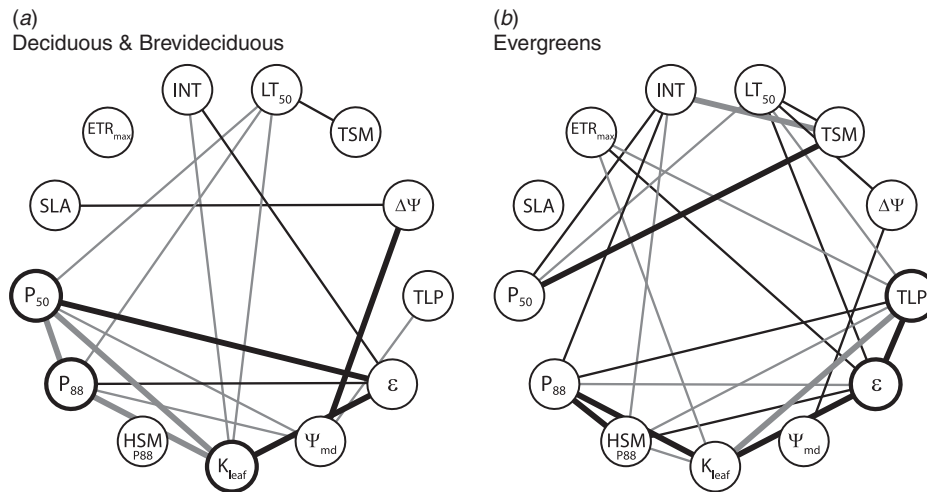
Fig. 5. Correlation networks for (*a*) deciduous-brevideciduous and (*b*) evergreen species leaf traits. Hydraulic traits: $\Delta\Psi$, daily difference in water potential; TLP, turgor loss point; ε, modulus of elasticity; Ψ_{md}, midday water potential; K_{leaf}, hydraulic conductance; HSM_{P88}, hydraulic safety margin at P_{88}; P_{88}, water potential at 88% loss of conductivity; P_{50}, water potential at 50% loss of conductivity. Anatomical and photosynthetic traits: SLA, specific leaf area; ETR_{max}, photosynthetic capacity measured as maximum electron transport rate. Low temperature resistance traits: INT, ice nucleation temperature; LT_{50}, leaf lethal temperature; TSM, thermal safety margin. Grey and black lines indicate positive and negative correlations respectively. Correlation strength is represented by lines thickness. Only significant correlations with r > 0.6 are shown. Traits identified by black circles show the highest centrality value in terms of weighted degree (the sum of all the significant coefficients of correlation of a node). For correlation coefficients, see Table S1, available as Supplementary Material to this paper. Values of INT, LT_{50} and TSM for *Lonchocarpus muehlbergianum* species were excluded from the correlation network (*a*).

between INT and HSM_{P88}, whereas such correlations were not found in deciduous and brevideciduous trees (Table S1). In both networks, SLA was weakly or not related to other traits.

Discussion

Our results show that evergreen and deciduous–brevideciduous species have different adaptations to cope with the consequences of subzero temperatures and water deficits. Deciduous species in our study had shorter leaf lifespan and were more efficient in leaf water transport (higher K_{leaf_max}), and showed higher photosynthetic capacity (ETR) than evergreens. These traits are linked to an acquisitive strategy, which implicate fast assimilation of CO_2 during the favourable season and, consequently, exhibition of a strong seasonal pattern of growth (di Francescantonio *et al.* 2018). Evergreen species, on the other hand, had the opposite behaviour, with a longer leaf lifespan, lower ETR and K_{leaf_max}, and denser wood. These traits have been associated with a conservative strategy in the use of resources (Wright *et al.* 2004; Chave *et al.* 2009; Reich 2014), reflected in a less pronounced seasonal growth patterns throughout the year (di Francescantonio *et al.* 2018).

SLA was of minor importance in the present study. Contrary to relationships among SLA, and TLP, P_{50} or K_{leaf} found by other authors (e.g. Nardini *et al.* 2012; Simonin *et al.* 2012; Villagra *et al.* 2013), we found no significant correlations between SLA and other physiological traits. Similarly, veins density was not related to phenological

patterns or stress resistance as found in other studies (Sack and Frole 2006; Nardini *et al.* 2012; Sack and Scoffoni 2013). This trait was very variable among species. For example, members of the Fabaceae family, such as *P. rigida* and *H. balansae*, have bipinnately compound leaves with a comparatively high density of veins that complicate the interspecific functional relationships (Fig. S6).

Contrary to our expectations, deciduous and brevideciduous species were less vulnerable to water stress events (higher HSM_{P88}) than evergreen species. However, this margin was very small. Considering that air saturation deficits occurring in the region during summer can be as high as 4.0 kPa (di Francescantonio *et al.* 2018), more resistant leaves could help deciduous species to sustain high carbon assimilation and growth rates even under the least favourable conditions. We also found that deciduous and brevideciduous species show a positive relationship between leaf P_{50}, P_{88} and K_{leaf_max}, (i.e. less drought-resistant species transport water more efficiently), suggesting the existence of a trade-off between the investments in construction of foliar tissues specialised in water transport and traits that provide resistance to water deficit in these groups of species. This trade-off was not observed in evergreen species. In this case, species with higher hydraulic conductance tended to have more negative P_{88} values (i.e. were more resistant). This could be due to the low values of hydraulic conductance of evergreens, particularly for three of the four species studied, which are consistent with other trait values typical of a conservative strategy (e.g. low photosynthetic capacity).

Oliva Carrasco *et al*. (2015) showed that for the same pool of species that the safety margins calculated as the difference between minimum leaf water potential and the stem xylem pressure at the catastrophic hydraulic dysfunction level ($P_{88 \text{ branch}}$) were related to sapwood capacitance in an asymptotic way. In their study, all species except the brevideciduous *P. rigida* and *L. muehlbergianum* had positive safety margins for branches. In the present study, we obtained positive safety margins for leaves of most deciduous and brevideciduous species, but for evergreens the values were negative. Evergreen species could significantly lose hydraulic conductance on a daily basis, especially during dry spells. Negative values in leaf safety margins should be relatively common (Johnson *et al*. 2016), and consistent with the hydraulic vulnerability segmentation hypothesis. For species with high wood density (i.e. with high construction costs) and low sapwood capacitance, such as evergreens in the present study, it would be reasonable to expect disposable leaves (negative leaf safety margins) to protect expensive stems from hydraulic failure.

Differences in the modulus of elasticity (ε) could also explain differences between phenological groups. This trait represents the elasticity – or its inverse, rigidity – of the leaf tissues (Bartlett *et al*. 2012). Thus, ε is regarded as a key leaf trait because it is functionally related to the capacity of the leaf tissue to resist drought events through cell water conservation (Salleo *et al*. 1997; Niinemets 2001), tolerate subzero temperatures (Scholz *et al*. 2012; Zhang *et al*. 2016), and, more recently, has been related to the rate of CO_2 assimilation (Nadal *et al*. 2018). We found that deciduous species had more elastic leaf tissues than evergreen species, and that the elasticity of the tissues was related to the capacity of water transport in the leaves. Further, we observed that deciduous species with more elastic leaf tissues (lower ε) were those with lower resistance to hydraulic dysfunction (higher P_{88} and P_{50}). In evergreen species, in contrast, an inverse relationship between ε and P_{88} was found, and as observed in global datasets, a negative relationship was found between TLP and ε (Bartlett *et al*. 2012), proving its close association with a species' level of drought tolerance. Evergreens also showed a negative correlation between cold damage (LT_{50}) and ε. This could be because more rigid cell walls increase resistance to ice formation on leaves since they confer greater mechanical resistance to the pressure exerted by ice in extracellular spaces (Yamada *et al*. 2002; Scholz *et al*. 2012; Arias *et al*. 2015).

Deciduous and only one brevideciduous species exhibited freezing avoidance (INT ~ LT_{50}), whereas evergreen species were freezing tolerant (IN*T* < LT_{50}). Evasion mechanisms, unlike tolerance, can only be effective for a few hours (Goldstein *et al*. 1985; Rada *et al*. 1987; Sierra-Almeida *et al*. 2009). For Atlantic Forest species, both mechanisms are probably equally effective because low temperatures and freezing periods are relatively short in duration. We found a strong negative correlation between wood density and INT. A possible explanation is that denser woods have less water content than light woods and thus less favourable conditions to ice formation that could propagates into leaves through vascular tissues (Hacker and Neuner 2007).

In contrast, denser wood have small diameter xylem vessels that are more resistant to embolism formation due to freezing-thaw cycles compared with lighter wood species (Lintunen *et al*. 2013). This relationship between wood density and ice nucleation temperature indirectly reflect the life history strategies of the species aimed at stress tolerance, meaning that species with higher wood density and longer LL are less frost susceptible species.

Within this group of evergreen species, a negative correlation between INT and P50 and a positive correlation between INT and HSM_{P88} suggest that there is trade-off in frost and drought tolerance. As previously mentioned, within evergreens, hydraulic conductance was higher in less drought susceptible species. The higher water content in leaf tissues could make them more susceptible to ice nucleation resulting indirectly in the observed trade-off between drought and frost resistance. A greater number of species need to be studied to further understand these trait associations and trade-offs within phenological groups.

The semi-deciduous Atlantic Forest constitutes the southern range limit of many species, which is reflected by the decline of tree species diversity at a regional scale from north to south (Oliveira-Filho *et al*. 2015). In this context, low temperatures may act as an evolutionary selective factor for some of the most frost-sensitive species limiting its distribution as occurs, for example, to the tropical palm *Euterpe edulis* (Gatti *et al*. 2008). If the occurrence of episodic spring or late frost events increases in intensity and duration as predicted for the area (Pizarro *et al*. 2013; Scarano and Ceotto 2015), this could have considerable ecological implications, and species with avoidance mechanisms, such as the deciduous and brevideciduous species in the present study, would be the most affected.

In summary, deciduous species presented relatively high values of safety margins, allowing them to sustain photosynthetic activity during the growing season. These species did not have hydraulic segmentation (Oliva Carrasco *et al*. 2015) and avoid low temperatures, both mechanisms consistent with their winter deciduousness. The absence of a dry season is likely to promote greater phenological diversity, increasing the possibility of intermediate iso- or anisohydric behaviour (Braga *et al*. 2016) and probably explains the weak relationship between leaf phenology and isohydricity (Fig S4). Thus, brevideciduous species exhibited hydraulic segmentation as evergreens, but also other hydraulic traits such as the safety margin that were similar to the deciduous species. Strategies for resistance to subzero temperatures were shared by both leaf habits. In contrast, species with evergreen leaves were damaged and frozen at more negative temperatures, so were more susceptible to water deficit than deciduous species. The significant loss of leaf hydraulic conductance observed in evergreens may facilitate the reduction of freezable water in tissues, and thus delay freezing under cold conditions (Arias *et al*. 2017). Considering all this, even though resistance to freezing and drought may share similar physiological mechanisms, adaptations must be studied within each leaf habit.

Further research is needed to elucidate resistance strategies to stress factors at whole-tree and stand levels. Resistance

strategies, possible links between hydraulic safety and efficiencies, and carbon economy among the different phenological groups (including more species), and especially the adaptive role of wood density and elasticity within the complex of drought-frost tolerance traits require further investigation. In a climate change scenario, this will lead to a better understanding of the mechanisms of adaptation and enable prediction of interspecific variation in species' responses to future changes in environmental conditions or extreme abiotic events in the subtropical Atlantic Forest.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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