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The Case of Leaf Cuticle and Leaf Dry Mass per Area

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PREDICTABILITY OF LEAF MORPHOLOGICAL TRAITS FOR PALEOECOLOGICAL RECONSTRUCTION: THE CASE OF LEAF CUTICLE AND LEAF DRY MASS PER AREA

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Our power to predict the future relies on our knowledge of the past. Paleoproxies are a powerful tool for understanding environmental and ecological conditions and changes across different time periods. However, constructing a functioning paleoproxy requires a well-constrained and robustly tested model. This is challenging, especially if ecological traits are involved. In the current study, we constructed an extended data set to test the reliability of the derivation of leaf dry mass per unit area (LMA) from the thickness of fossil gymnosperm cuticle. Specifically, we tested whether different leaf types (broad leaves, needles, and scales), intraspecific variability in cuticle thickness, and growing conditions affect the functioning of the proxy. Taxonomic groups were analyzed to uncover the possible taxonomic influence on LMA, cuticle thickness (CT), and the LMA-CT relationship. Our results indicate that the CT versus LMA relationship depends on multiple factors that can have various and incongruous effects on this relationship, depending especially on leaf type and growing conditions. We conclude that CT measured from gymnosperm fossils could be used as a proxy for LMA in past ecosystems for some broad- and scale-leaved gymnosperms but not for needle-leaved gymnosperms. However, caution must be taken when comparing species from different environments or growth conditions.

Keywords: gymnosperm, leaf mass per area (LMA), cuticle thickness (CT), paleoproxy, proxy, light.

Online enhancements: supplemental tables and figures.

Introduction

Paleoproxies are a potentially powerful tool for understanding past changes in the Earth’s climate, atmospheric conditions, and ecosystems (Lomax and Fraser 2015) and for linking these changes with long-term dynamics of ecosystem functioning (Tibby et al. 2016; Soh et al. 2017). Reconstructing past environmental conditions based on the paleontological record can be further confirmed using paleoproxies that indicate changes in ecosystem properties that are known to have specificities of species-specific effects on this relationship, depending especially on leaf type and growing conditions. We conclude that CT measured from gymnosperm fossils could be used as a proxy for LMA in past ecosystems for some broad- and scale-leaved gymnosperms but not for needle-leaved gymnosperms. However, caution must be taken when comparing species from different environments or growth conditions.

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Paleoproxies are a potentially powerful tool for understanding past changes in the Earth’s climate, atmospheric conditions, and ecosystems (Lomax and Fraser 2015) and for linking these changes with long-term dynamics of ecosystem functioning (Tibby et al. 2016; Soh et al. 2017). Reconstructing past environmental conditions based on the paleontological record can be further confirmed using paleoproxies that indicate changes in ecosystem properties that are known to have specific effects on taxonomic shifts. For example, calibration models of species-specific variability in stomatal density are well-established proxies for interpreting past clastic changes and how the variability was related to changes in atmospheric CO2 levels (Royer et al. 2001; Beerling and Royer 2002).

Plants act as both monitors and recorders of environmental changes, meaning that when comparing plant fossils with the traits of living individuals of the same species (or nearest living relatives or nearest living ecological equivalents; Chaloner and McElwain 1997), it is possible to construct mechanistic paleoproxies (Lomax and Fraser 2015). Thus, paleoproxies can be used to reconstruct mean annual or growth season temperatures, precipitation, CO2 levels, and so on (Chaloner and McElwain 1997; Uhl et al. 2003, 2007; Haworth and McElwain 2008; Bacon et al. 2016). Paleoproxies focusing on plant data have mainly addressed stomata and leaf margins from fossilized leaves and pollen extracted from sediments (Chaloner and McElwain 1997; Uhl et al. 2007).

However, there are important caveats in the use of paleoproxies, and the validity of some has been passionately debated. For example, leaf margin analysis can considerably over- or underestimate the mean annual temperature if there is a small taphonomic bias (Uhl et al. 2003). Meanwhile, other methods may give information only about specific environmental factors;
for example, leaf venation density concurs with environmental conditions changing transpiration or water availability but not CO₂ (Uhl and Mosbrugger 1999). So some authors have argued against calibrating proxies with modern vegetation (Uhl et al. 2007). However, when several separate methods are used to describe the paleoenvironment (e.g., leaf physiognomic methods, fossil plant and animal records, and the nearest living relative method) and give similar results, the reliability of these results increases (Uhl et al. 2007). Nevertheless, to achieve this, the proxies have to be comprehensively tested (Lomax et al. 2012; Tibby et al. 2016). However, even when no quantitative information can be obtained for climatic changes in the past, the detection of a substantial climatic change with a proxy gives valuable information (Uhl et al. 2007; Soh et al. 2017). Therefore, it is necessary to develop and test paleoproxies for comparing, confirming, and possibly specifying results (e.g., whether the changes in population composition are also accompanied by changes in plant functional traits).

Constructing a proxy based on ecologically responsive traits is difficult, as ecological relationships are known to escape attempts to form laws and steadfast patterns (Lawton 1999). However, some of the complications present for paleoproxies can be overcome by additional data or calculations. For example, the use of stomata to infer paleo-CO₂ is complicated by the fact that the density of stomata is heavily influenced by water availability during leaf expansion. One resolution is to use the stomatal index or calculations that include the size of epidermal cells to account for this issue (Royer 2001).

**Cuticle Thickness and Leaf Dry Mass per Area: Relationship to Each Other and the Climate**

Leaf dry mass per area (LMA) is one of the most studied plant functional traits, while cuticle thickness (CT) is much less researched. However, both can relay information about plant function and ecological strategies. For example, LMA is an important functional trait expressing plant investment in leaves (Wright et al. 2004). Further, there are fundamental trade-offs between plant functional traits, including LMA, that make up the global plant form and function spectrum (Díaz et al. 2016) or, specifically, the leaf economic spectrum (LES; Wright et al. 2004). These spectra tell us that LMA is negatively correlated with leaf nitrogen content (Díaz et al. 2016) and positively correlated with leaf longevity (Wright et al. 2004). From there, further assumptions can be made about net assimilation rates and other physiological traits (Wright et al. 2004, 2005), as well as ecological strategies (Westoby et al. 2002). LMA components, leaf thickness and density, can give additional physiological and ecological information if they could be inferred from a proxy. Thick leaves can mean more photosynthetic tissue resulting in higher assimilation and indicate that the leaves have been growing in full sunlight (Niinemets 1999; Xiao et al. 2011; Tosens et al. 2012a). At the same time, high leaf density can result from high cell wall thickness producing low assimilation rates (Hassiotou et al. 2009; Tomáš et al. 2013), while both thick leaves and high leaf density can contribute to high LMA (Niinemets 1999). Even though LMA and other leaf functional traits have been found to be largely independent of climate on a global scale (Wright et al. 2004), on a local scale, the relationship between LMA and CO₂ concentration is well established (Ainsworth and Long 2005; Poorter et al. 2009; Bacon et al. 2016). Furthermore, understanding general patterns of LMA variation in a geological timescale could still provide us with valuable insights into the general tendencies of vegetation responses to large-scale climatic and atmospheric changes.

CT can give valuable information on the past environment as well. Plants with thicker cuticles may be reducing cuticular transpiration, which suggests an acquisitive strategy in water-limited conditions if a thick epidermis is present (reducing water loss for maximum growth efficiency; Aasamaa et al. 2001) or a conservative strategy, in which a plant increases investment in protective tissues for abiotic and biotic defenses in long-lived leaves (Hanley et al. 2007). Similarly, if CT increases in geological time in an ecosystem, an increased pressure from herbivory or water limitation may be inferred (Soh et al. 2017).

Recently, Soh et al. (2017) proposed a new paleoproxy based on LMA and CT. The report claims that by measuring CT in fossilized samples of extant gymnosperms, it is possible to estimate the LMA of these leaves. LMA, in turn, reflects major climate change events in the past (specifically for the Triassic–Jurassic transition). This estimation of LMA from CT—hereafter, the “paleo-LMA proxy”—could also indicate changes in ecosystem properties that concur with climatic changes, as LMA is known to be higher at high CO₂ levels and lower at low CO₂ levels (Ainsworth and Long 2005; Poorter et al. 2009; Bacon et al. 2016). Specifically, LMA provides a fairly simple way to investigate ecosystem function (Wright et al. 2004; Bacon et al. 2016), while it also responds to climatic conditions—together allowing exploration into how plants have reacted to major climatic changes (Royer et al. 2010; Blonder et al. 2014; Haworth and Raschi 2014; Bacon et al. 2016). Understanding the changes and differences in LMA, which cannot be directly measured from paleontological material (Royer et al. 2007), in the geological timescale could provide us valuable insight into changes both in the past and in the future.

The paleo-LMA proxy proposed by Soh et al. (2017) is based on 20 broad-leaved gymnosperm species (15 genera, 8 families) sampled from four botanical gardens and universities in Australia and Ireland. These samples show a strong relationship between LMA and CT among extant gymnosperms (Soh et al. 2017). Although a range of broad-leaved taxa is covered in their data set and their results compared well with previously established proxies (Soh et al. 2017), a larger sample is needed to confirm their results, while other foliage morphologies should be tested to uncover whether the LMA-CT relationship stands in needles and scales as well. In this analysis, we examine the paleo-LMA proxy from different angles (leaf type, leaf position in the canopy, and growth conditions).

**Sources of Variation in the LMA-CT Relationship in Gymnosperms**

There is variability in LMA and CT—between species and between individuals of the same species (Leng et al. 2001; England and Attiwill 2011). This variance comes from both ontogeny
and evolutionary history, but their relative proportions and patterns related to ecosystem properties are so far largely unknown (Laanisto et al. 2008; Albert et al. 2011; Siefert et al. 2015). This is a major obstacle for building paleoproxies, as even the variability within one species through a relatively short timescale in one region has to be carefully considered when constructing a paleoproxy (Tibby et al. 2016). In addition, multispecies paleoproxies exhibit an additional layer of variability problems because of the variation in fossilization processes (Spicer 1989; Locatelli et al. 2016). So several taxonomic groups need to be analyzed to uncover the possible taxonomic influence on LMA, CT, and the dependence of the LMA-CT relationship on taxonomic affinity.

Further, the paleoproxies estimating LMA have so far focused only on broad-leaved taxa (Royer et al. 2007; Soh et al. 2017). Despite being a relatively small group (14 families with 1088 accepted species), gymnosperms have highly variable leaf morphology and functionality (Brodribb et al. 2010). Because of a long and diverse evolutionary history and biogeography, gymnosperms represent four out of the five main lineages of seed plants: cycads, ginkgos, gnetophytes, and conifers (Wang and Ran 2014). Gymnosperms have experienced frequent morphological diversification, including parallel and convergent evolution during the past 300–350 Myr, resulting in extreme differences in leaf type and distribution (Wang and Ran 2014).

Functional and structural differences in gymnosperms are also expressed within species not only between species. Intraspecific variability of leaf morphological and functional traits is one of the most common adaptations that increases individual fitness in variable environments across the species’ range (Albert et al. 2010; Anderegg 2015). For example, cuticles of some pines growing in higher altitudes are thicker than those of the lower-altitude individuals, increasing the resistance of needles to abrasion from high wind speeds (Shepherd and Griffiths 2006). Leaf morphological traits in gymnosperms, including cuticle structure and thickness, strongly vary in dependence on environmental conditions (e.g., amount of light and moisture; Niinemets et al. 2015) and with the plant ontogeny and leaf age (Niinemets et al. 2016; Kuusk et al. 2018). Within-species differences in plants manifest more prominently between populations, including gymnosperms (Anderegg 2015), which is why sampling across populations with diverse environmental conditions is a crucial step in gathering information about the potential variability patterns within and between traits (Fajardo and Piper 2011). The main goal of our study is to analyze the LMA-CT relationship based on an extended data set (86 species from 62 genera, 11 families) of CT and LMA measurements from extant gymnosperms partly overlapping with Soh et al. (2017) and to test this relationship from several ecological angles. Specifically, we test the predictability of the paleo-LMA proxy (1) between extant gymnosperms with different types of leaves (broad leaves, needles, and scales), (2) with the effect of limited growth irradiance, (3) across diverse gymnosperm families, and (4) in different growing conditions. We aim to introduce a large data set of gymnosperm CT and LMA values to provide data on extant species for comparison with fossil cuticles and with the inference of the paleo-LMA proxy. Further, we aim to provide morphological and environmental factors influencing this relationship to be considered when applying the proxy.

**Material and Methods**

**Plants**

We measured healthy current-year leaves from 86 gymnosperm species (62 genera, 11 families) originating from the Botanical Garden at the Natural History Museum of Denmark (University of Copenhagen) in Copenhagen, the University of Tasmania, and several nurseries in mainland Australia (table A3; tables A1–A3 are available online). The data set included 50 broad-leaved (11 species and 13 genera overlapping with Soh et al. 2017) and 11 needle-leaved species, 1 species with a phylloclade, and 24 species with scales (for leaf types and native habitats, see table A3). A phylloclade is a flattened photosynthetic shoot of Phyllocladus asplenifolius (Labill.) Hook. in this sample. Because it performs the function of a leaf, it is included in the overall analysis between CT and LMA but not included in any of the analyses based on leaf type.

Healthy, fully expanded, nonsenescent leaves were chosen for the experiment. The plants from the Botanical Garden at the Natural History Museum of Denmark were grown in a high-humidity palm house with natural light supplemented with additional LED lamps. The leaves were sampled at three different light levels within the canopy (full light: 1200 μmol m⁻² s⁻¹; semishade: 500 μmol m⁻² s⁻¹; shade: 150 μmol m⁻² s⁻¹) to test how the LMA-CT relationship is modified by growth irradiance. Light conditions across the canopy were measured repeatedly throughout the day over 7 d with a Li-Cor Li-250A light meter (Lincoln, NE) to find the leaves growing in the specified light conditions (±50 μmol m⁻² s⁻¹).

The species sampled at the University of Tasmania were grown in two different environments: tropical species were grown in controlled conditions in a glasshouse with 25°C/16°C day/night temperatures, while higher-latitude species were grown under natural temperature conditions in a mesh greenhouse. Leaves exposed to full sunlight were studied in all cases. Plants were watered regularly and repotted routinely in soil with a slow-release fertilizer. Air humidity for both settings was between 55% and 100% (for details, see Veromann-Jürgenson et al. 2020). Therefore, the temperature and humidity were within the natural ranges for all the studied species (table A3).

The cycad samples collected from Australian nurseries were native species or species adapted to similar environments potted or planted outside under natural conditions but watered and fertilized regularly to ensure optimal growth conditions.

The names of the species were standardized, and their systematics are in accordance with the Plant List (http://www.theplantlist.org/) and Leslie et al. (2012). The natural growth conditions and habitat were selected according to Eckenwalder (2009) for conifers and the IUCN Red List (https://www.iucnredlist.org/) and the Useful Tropical Plants Database (http://tropical.theferns.info/) for other species.

**Cuticle Thickness**

Sample preparation for light microscopy followed Tosens et al. (2012b), and cuticle staining and thickness measurements followed Soh et al. (2017). Small foliage samples approximately
6 mm long and 4 mm wide (or the width of the needle or scale) were cut from intercostal areas of healthy, fully matured leaves and fixed in fixation buffer (3% glutaric aldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 6.9) under vacuum in a syringe. The samples were postfixed, dehydrated, and embedded in LR white resin (Electron Microscopy Sciences, Hatfield, PA). Cuticle is impervious to physical and chemical changes (Evert 2006; Soh et al. 2017), so it is unlikely that the CT was affected by sample preparation. Cross sections 1 μm thick were prepared by an ultramicrotome (Leica EM UC7, Leica, Vienna, Austria) for light microscopy and stained with toluidine blue. As the cuticle is mainly composed of insoluble cutin, cutan, and soluble waxes (Evert 2006; Soh et al. 2017) and is less acidic than the epiderm, it is lighter blue on the sections (fig. 1, smaller panels) and can be differentiated from the other tissues. Care was taken to have ideally perpendicular cross-sectional cuts through the leaves. A second method, Sudan III staining, was used for validating the chosen methodology. Three species were chosen from each leaf type representing a thin, average, and thick cuticle based on the toluidine blue staining. The sections were viewed in bright field with a Nikon Eclipse E600 microscope (Nikon, Kyoto, Japan) and an EVOS FL Auto 2 (Thermo Fisher Scientific, Waltham, MA) with phase contrast at ×400 magnification and photographed with a 5-MP Nikon DS-Fi1 digital microscope camera and the EVOS FL Auto 2. Figure 1 presents a selection of leaf sections, where the darker blue lines are minimal folding of very thick cuticles (fig. 1C). CT was not measured over the folds.

CT (average cuticle thickness over the upper and the lower cuticle is hereafter referred to as “CT,” while upper and lower

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**Fig. 1** Light microscopy sections of extant broad-leaved gymnosperms illustrating the variability of lower and upper cuticle thickness (CT) at different leaf dry mass per area (LMA) values. Sections were stained with toluidine blue. Cuticles embedded in epidermis, scleroids, and mesophyll are shown with arrows. Scale bars = 50 μm.
CT will be specified as such) was measured in each sample at 10 locations for both the upper (typically abaxial for tree species) and the lower (typically adaxial) surfaces at the thickest point between the epidermal cell surface and the outer layer of the cuticle (Soh et al. 2017) because a notable proportion of a cuticle can be embedded between epidermal cells (fig. 1C; Onoda et al. 2012). The vicinity of stomata and guard cells, where the cuticle can be nonproportionally thick, was avoided. The measurements were then averaged. The ratio of average upper CT to average lower CT was also calculated. The two staining methods gave highly similar results (fig. A1; figs. A1–A3 are available online; e.g., overall the difference was ±4.9%, with the smallest difference for thick leaves; measurements from the Sudan III staining gave a 3.8% smaller thickness on average) and the biggest difference for scales (Sudan III staining gave a 5.5% bigger thickness on average).

Leaf thickness was measured with ImageJ version 1.48 software (Wayne Rasband/National Institutes of Health, Bethesda, MD) from the top edge of the upper cuticle to the bottom of the lower cuticle in 10 locations, avoiding leaf venation and stomata in each sample, and then averaged.

**Leaf Dry Mass per Area**

After taking the samples for cuticular analysis, the same leaf, needle, or scale fractions were scanned at 300 dpi, then oven dried at 70°C for 48 h to determine their dry mass. Leaf area was defined as projected area determined from scanned images with ImageJ software. For nonflat needle- and scale-leaved foliage, light microscopy photographs were used to measure foliage width and, when pertinent, the side length (e.g., for species with triangular or rhomboidal cross section) from the cross sections at the center of the foliage element (anatomical sampling and analyzing protocol was conducted according to Tosens et al. 2012a). Needle length was measured from scanned images, and needle cross-sectional area was calculated according to Niinemets et al. (2002). From these measurements, the leaf density was calculated as in Poorter et al. (2009).

**Statistical Analyses**

Statistical analyses were performed with Statistica version 10.0 (StatSoft, Tulsa, OK). Analysis of covariance (ANCOVA), regression analyses, and t-tests were used to analyze the relationships between traits, as all continuous traits had a normal distribution according to the Kolmogorov-Smirnov test. Data were grouped by leaf type, origin of the sample, order, and family, and the samples collected from the Botanical Garden at the Natural History Museum of Denmark were grouped by growth light availability, or different climatic conditions) were also performed by adjusting P values with the Sidak correction. These analyses were run with the R package SMATR (Warton et al. 2012).

Cross validation was used to test the ability of CT to predict LMA. First, a linear model was generated including all the data points in the data set so that single leaf-level measurements not averaged at the species level were the experimental units (n = 195), then K-fold cross validation was used to assess linear model predictive ability (Maindonald and Braun 2015). The data were randomly assigned to 10 bins. At each iteration, one bin was removed, while the remaining data were used to refit the regression model and to predict the deleted observations. The same analysis was separately carried out for each leaf type, but in this case, the data were assigned to five bins for leaves and scales and three for needles because of the different sample size of each group compared with all data pooled. Cross validation was implemented through the function CVlm included in the R package DAAG (Maindonald and Braun 2015). R version 3.53 (R Foundation for Statistical Computing, Vienna, Austria) was used for the analysis. The root mean square error (RMSE), which is in the same units as the response variable (i.e., LMA), was calculated and allows estimation of the average extent of uncertainty (percent) of model prediction. RMSE was combined with the maximum and minimum values of LMA for extant evergreen gymnosperms (∼500 and 100 g m⁻²; Poorter et al. 2009) to test the model uncertainty at the low and high ends of gymnosperm LMA spectrum.

**Results**

**LMA versus CT Relationship: General Patterns**

LMA varied tenfold from 39.1 ± 3.7 g m⁻² (average ± SE) to 410.6 ± 11.9 g m⁻², and average CT varied sixfold from 2.77 ± 0.16 µm to 17.8 ± 3.5 µm across the samples (fig. 1). When all data were pooled, the relationship between LMA and CT was weaker (slope = 9.54 g m⁻² µm⁻¹; R² = 0.20; P < 0.05; fig. 2) than the log transformation (slope = 0.54; intercept = 3.94; R² = 0.27; P < 0.05). The strength of the relationship depended on leaf type. A significant relationship was found in gymnosperms with flat leaves and scales, but no relationship was found in needle-leaved species (fig. 2).

The results of 10-bin cross validation showed a weak relationship between observed and predicted LMA (slope = 0.20; intercept = 132.8 g m⁻²; R² = 0.18; P < 0.05; fig. 3). The RMSE of the model LMA-CT relationship for all data pooled was 66.7 g m⁻² after cross validation. The magnitude of RMSE was similar within each leaf type (leaf = 80.56; scale = 58.34; needle = 63.25). However, the sample size of each group was relatively small (especially for scales and needles) to run a
cross-validation analysis, so the RMSE of each group was not considered in further analyses. If we associate the RMSE obtained for all data pooled with the extremes of LMA values reported for gymnosperms, the LMA uncertainty is 67% for an average species predicted to have an LMA of 100 g m\(^{-2}\). The uncertainty increases with decreasing LMA. For an extreme LMA of 500 g m\(^{-2}\), the uncertainty is predicted to be 13%.

The upper and lower CT varied 4-fold and 4.4-fold, respectively. Analysis into CT showed that the upper and lower CT correlated strongly (\(R^2_p = 0.46\); \(P < 0.0001\)).

CT correlated positively with LMA constituents, leaf density and leaf thickness. However, there was wide variability in both density (\(R^2 = 0.045\); \(P = 0.028\)) and thickness (\(R^2 = 0.077\); \(P = 0.0057\)), so the correlations were very weak, although significant likely due at least in part to the large sample size. The log transformation improved the correlations slightly (fig. A2). Leaf shape influenced the dependence of leaf thickness and density on CT. Specifically, leaf thickness correlated positively with CT in leaves but not in needles and scales, while density depended on CT in scales but not in other leaf shapes (table 1). Furthermore, the sampling site influenced the leaf thickness and density relationship with CT, where both leaf thickness and density correlated positively with CT in samples collected from the Botanical Garden at the Natural History Museum of Denmark but not in samples from mainland Australia. When all data were pooled, LMA correlated positively albeit weakly with its components: leaf thickness (\(R^2 = 0.23\); \(P = 0.018\)) and density (\(R^2 = 0.30\); \(P = 0.026\)).

A further analysis into species collected from the Botanical Garden at the Natural History Museum of Denmark revealed that growth light conditions affect the relationship between CT thickness and CT density, in which thickness depended on CT in all light conditions but density depended on CT only in full light and not in semishade or shade (table 2).

**Variation in LMA versus CT Relationship in Dependence on Environmental Conditions, Site, Leaf Form, and Taxonomic Grouping**

The predictability of the paleo-LMA proxy across a light gradient was tested with nine species of Cycadales sampled in light, semishade, and shade conditions in the Botanical Garden at the Natural History Museum of Denmark (see “Material and Methods”). Overall, there was a positive relationship between LMA and CT (fig. 4A), although the strength of the relationship depended on growth light, and it was strongest for leaves grown in full light. Average LMA did not significantly differ between the three light environments, while the thickness of the cuticle on leaves grown in full light was significantly larger than the cuticle on leaves grown in semishade or shade (fig. 4B).

Similarly, LMA depended on the thickness of the upper (\(R^2 = 0.56\); \(P < 0.0001\); log-transformed \(R^2 = 0.56\); \(P < 0.0001\)) and the lower (\(R^2 = 0.45\); \(P < 0.0001\); log-transformed \(R^2 = 0.33\); \(P = 0.001\)) cuticle overall. Furthermore, the relationship was also significant in leaves grown in full light for

**Fig. 2** Leaf dry mass per area (LMA) dependence on average cuticle thickness (CT). LMA dependence on CT across the sample among species with broad-leaved, scale-shaped, and needle-shaped foliage. A phylloclade is a broadened photosynthetic shoot of *Phyllocladus asplenifolius* (Labill.) Hook. in this sample. Because it performs the function of a leaf, it is included in the overall analysis between CT and LMA but not included in any of the analyses based on leaf type. Axes are log\(_{10}\) scaled. Error bars show average ± SE of all presented species (\(n = 3\)). Data were fitted by linear regression (relationships are significant at \(P < 0.05\)).
both the upper CT \((R^2 = 0.62; P = 0.0069; \log\text{-transformed} R^2 = 0.66; P = 0.0047)\) and the lower CT \((R^2 = 0.75; P = 0.0016; \log\text{-transformed} R^2 = 0.50; P = 0.019)\).

CT depended on family; for example, the lowest CT was in Pinaceae (needle leaves) and Stangeriaceae (broad leaves; fig. 5). In families dominated by needle-leaved species, such as Pinaceae and Taxaceae, LMA and CT values were not significantly correlated \((R^2 = 0.007; P > 0.05)\). LMA and CT were positively correlated in families dominated by broad-leaved and scale-leaved species (e.g., Zamiaceae and Podocarpaceae; \(R^2 = 0.31; P = 0.0004\)). Both the original data and the log-transformed data for upper and lower CT followed the average LMA-CT relationship (table A2).

LMA-CT relationship also depended on the sampling site (fig. 6). The correlation was strongest for species studied at the Botanical Garden at the Natural History Museum of Denmark. Notably, these species were all broad-leaved. Differences between the slopes of LMA-CT relationships were tested separately for leaf type, light availability, climatic conditions, and taxonomic affinity (figs. 2, 4A, 6; table A1). The LMA-CT slope was not significant for needles, and it was only marginally significant for leaves grown at low light. Conversely, climatic

### Table 1

| Cuticle thickness correlations with thickness and density depending on humidity preference, leaf shape, and origin of the sample |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Leaf shape                      | Origin of sample                        |
|                                | Botanical Garden at the Natural History Museum of Denmark | Mainland Australia | University of Tasmania |
| (n = 50)                        |                                               |                  |                  |
| Needle (n = 11)                 |                                               |                  |                  |
| Scale (n = 24)                  |                                               |                  |                  |
| Cuticle thickness               |                                               |                  |                  |
| vs. leaf thickness              |                                               |                  |                  |
| \(R^2 = .13, \quad P = .005\)  |                                               |                  |                  |
| \(R^2 = .16, \quad P = .11\)   |                                               |                  |                  |
| \(R^2 = .06, \quad P = .12\)   |                                               |                  |                  |
| Cuticle thickness               |                                               |                  |                  |
| vs. leaf density                |                                               |                  |                  |
| \(R^2 = .013, \quad P = .22\)  |                                               |                  |                  |
| \(R^2 = .03, \quad P = .31\)   |                                               |                  |                  |
| \(R^2 = .15, \quad P = .031\)  |                                               |                  |                  |

Note. The statistically significant correlations are underlined. Data were fitted with linear regression. Significant at \(P < 0.05\).
Table 2
Effect of Growth Light Conditions on the Cuticle Thickness-Leaf Thickness and Cuticle Thickness-Leaf Density Relationships

<table>
<thead>
<tr>
<th>Cuticle thickness vs. leaf thickness</th>
<th>Full light</th>
<th>Semishade</th>
<th>Shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>R² = .72, P = .0009</td>
<td>R² = .57, P = .007</td>
<td>R² = .82, P = .001</td>
<td></td>
</tr>
<tr>
<td>Cuticle thickness vs. leaf density</td>
<td>R² = .49, P = .017</td>
<td>R² = .15, P = .42</td>
<td>R² = .006, P = .0009</td>
</tr>
</tbody>
</table>

Note. Samples collected from the Botanical Garden at the Natural History Museum of Denmark (see “Material and Methods” for sampling and light conditions). Data were fitted with linear regression. The statistically significant correlations are underlined.

Discussion

LMA is a key plant trait characterizing plant functional strategies across environmental gradients (Wright et al. 2004) and providing insight into general ecosystem properties. However, LMA cannot be measured from fossilized leaf samples, and thus, attempts have been made to estimate it from different proxies in order to assess past ecosystem properties (Royer et al. 2007; Soh et al. 2017). Using CT to predict LMA is an attractive idea, as cuticles are more likely than mesophyll to be preserved through time. In the study of Soh et al. (2017), a proxy estimating LMA from CT was developed using 20 broad-leaved gymnosperm species from 15 genera. The current study greatly extends the broad-leaved data set, along with exploring two other leaf types (86 species from 62 genera), and tests the predictability of the paleo-LMA proxy from several ecological and evolutionary angles. Our data set represents a substantial proportion (8%) of all extant gymnosperms, making this the largest sample analyzing CT in gymnosperms. Even though there are only a handful of articles describing this trait in gymnosperms, our results are similar to previous work (fig. A3; Anfodillo et al. 2002; Harrington and Carlson 2015; Soh et al. 2017).

The Relationship of LMA with CT and the Predictability of the Paleo-LMA Proxy across Extant Gymnosperms and within Different Types of Leaves

The initial analysis of Soh et al. (2017) on broad-leaved gymnosperms indicated a strong relationship between CT and LMA (R² = 0.78). Our results were largely confirmatory of the proposed paleo-LMA proxy. However, in our analysis, the relationship between CT and LMA was variable between gymnosperm species with different leaf types (fig. 2). It is important to expand the studied leaf types, as the Royer et al. (2007) paleopropx estimating LMA from petiole width and leaf area is also calibrated only for broad-leaved species. Species with broad leaves or scales (e.g., from Cycadales, Cupressaceae, and Podocarpaceae) had the strongest relationships, although they were still weaker than the correlation found by Soh et al. (2017). Importantly, needle-leaved species, which make up the most abundant leaf functional group in gymnosperms, had no relationship between CT and LMA. When all groups were analyzed together, the relationship between CT and LMA was R² = 0.27, which could be considered a low predictability power to function as a proxy (Prairie et al. 1996).

Cross-validation analysis revealed considerable uncertainty in predicting LMA on CT across leaf types (fig. 3). The predictive power of the LMA-CT model is lower in cases of species with low LMA. For instance, a predicted LMA of 100 g m⁻² would bring an uncertainty of 67%. The variability of LMA in plants is more than 100-fold, and even though the highest LMA values are typically measured from evergreen gymnosperms, this group also has the highest variability among different plant functional types (Poorter et al. 2009). However, this overall model of gymnosperms still gives a rough estimate of LMA that can be used to infer, for example, a strong herbivory pressure or abiotic stress if mostly very high LMA is estimated for a community (Hanley et al. 2007; Soh et al. 2017). Furthermore, estimates of trends in ecological strategies could be made if further information is available from the fossil flora reconstructions or geological features, all the examined leaves are sun morphotypes, and the effect of changes in CO₂ concentration on LMA across the studied time period is considered (Ainsworth and Long 2005; Poorter et al. 2009; Royer et al. 2010; Bacon et al. 2016). Moreover, when only broad-leaved or scale-leaved gymnosperm samples from similar environmental conditions are explored, the paleo-LMA proxy could give even more reliable information.

Effect of Growth Irradiance on the Relationship of LMA with CT and the Predictability of the Paleo-LMA Proxy

Although the majority of fossilized leaves are sun morphotypes, especially for deciduous broad-leaved species (Spicer 1989; Soh et al. 2017), we can assume that the paleontological record contains shade leaves as well. Indeed, in some sediments, the share of sun and shade morphotypes is almost equal (Xiao et al. 2011). For evergreen and needle-leaved gymnosperms, their leaf-shedding dynamics are somewhat different, as the leaf shedding takes place throughout the year and the needles do not fall in the same pattern as broad leaves. Therefore, we cannot expect that the taphonomical processes in gymnosperms have an equally strong tendency toward higher probability of fossilization of full-light leaves—leaves from all canopy layers are known to successfully fossilize (Labe and Barate 1996). Further, from ecophysiological studies, we know that LMA of shade and sun leaves can be significantly different in both broad-leaved and needle-leaved trees (Duursma and Marshall 2006; Wyka et al. 2007; Catoni et al. 2015; Pugielli et al. 2017). However, it can be possible to distinguish sun and shade morphotypes (Xiao et al. 2011) and take this into consideration. Indeed, our analysis of nine broad-leaved cycad species growing in the Botanical Garden at the Natural History Museum of Denmark showed that the LMA-CT relationship depends on leaf position within the canopy (fig. 4). Upper canopy leaves exposed to full-light conditions had a much stronger LMA-CT relationship, while shade leaves exhibited lower correlations, mainly because of the high variability of CT among Zamiaceae.

Leaves sampled from different canopy heights also had different LMA-CT slopes—the likelihood-ratio test showed that, in contrast to full-light and semishade leaves, the LMA-CT slope...
for shade leaves was statistically insignificant (table A1). Such differences within a group of species suggest that individual variability of the LMA-CT relationship requires further research in the context of estimating the paleo-LMA proxy from fossilized cuticle measurements.

Therefore, examining assemblages of fossil leaves and, if possible, identifying and measuring only the cuticles of sun morphotypes for the paleo-LMA proxy could give higher confidence in its results. Further, the addition of another paleoproxy for the reconstruction of past environments can increase the reliability of the presently used proxies if they result in similar estimates for the paleoenvironment.

The Relationship of LMA with CT and the Predictability of the Paleo-LMA Proxy across Diverse Gymnosperm Families

Comparing CT and LMA correlations in different families (fig. 5) shows that affinity could play a significant role in shaping...
Fig. 5  Average cuticle thickness (CT) and leaf dry mass per area (LMA) across different gymnosperm families. CT differences are marked with lowercase letters, and different letters mark significant differences. CT in the four families marked with “a” was significantly larger than CT in families marked with “b” and “c.” Pinaceae and Stangeriaceae had the lowest average CT (marked with “c”). LMA differences are marked with uppercase letters. Whiskers represent standard errors.

Fig. 6  Relationships between cuticle thickness (CT) and leaf dry mass per area (LMA) measured in different climatic conditions (axes are log_{10} scaled). Data fitting is as in figure 2.
this potential paleoproxy. The functional form of the photosynthetic organs—leaf type—significantly affects the LMA-CT relationship (fig. 2), but these functional types are not consistently overlapping with the taxonomic affinity of the species. While some families such as Podocarpaceae and Zamiaceae that are dominated by broad-leaved species exhibit high correlations between CT and LMA, some such as Cupressaceae and Taxaceae do not. At the same time, needle-dominated Pinaceae shows the weakest relationship between CT and LMA.

These discrepancies can emerge as problems for paleoproxies in two different ways. First, there has to be further analysis on taxon-level variability in the dynamics of CT and LMA, which is still missing for the majority of gymnosperm species (Brodribb et al. 2010). Second, in order to make the paleo-LMA proxy reliable across different habitats, there has to be further analysis on how much CT and LMA vary in different environmental conditions. Without the synthesis and standardization of these two aspects, we are facing too much uncertainty for a paleoproxy to predict LMA with confidence. Consequently, the proxy should additionally regard the taxonomy if possible—extent relatives should be used for the relationship between CT and LMA, as was done by Soh et al. (2017).

The Relationship of LMA with CT and the Predictability of the Paleo-LMA Proxy in Different Growing Conditions

Preserved fossils are highly fragmented, especially in the case of plants, which have a modular nature. Therefore, plant fossils typically elude all attempts to reconstruct whole individuals because some parts such as leaves are abundant and can disperse long distances from the woody and underground parts (Spicer 1989). Thus, when leaf fossils of tall woody plants (such as gymnosperms) are studied, it could be difficult in some cases to establish the exact local ecological conditions in which the individual carrying a specific fossilized leaf grew, while in other cases, the leaf is entombed and associated with little transport (Burnham 1989; McCabe and Totman Parrish 1992).

Therefore, it is important to consider that there is a function-related variability in CT and LMA. For example, high-altitude pine species have a thicker cuticle to prevent frost-drought damage (Anfodillo et al. 2002) or wind abrasion injuries (Herrick and Friedland 1991). The current study showed that when CT and LMA were measured in different environmental conditions, the LMA-CT relationship was different irrespective of the measured genus (fig. 6; table A1). In essence, although all species from mainland Australia and Denmark were conifers, their LMA-CT relationships were contrasting. While individuals measured in mainland Australia and Tasmania showed very similar slopes in the LMA-CT relationship, measurements done in the Botanical Garden at the Natural History Museum of Denmark had a significantly different slope (table A1). Consequently, in order to make more accurate predictions of LMA based on fossil cuticles, the growth conditions need to be reconstructed if several locations are compared. An alternative is to focus on a smaller taxonomic or functional group within gymnosperms as Soh et al. (2017) did by inferring only the LMA of broad-leaved gymnosperms. The caveats and challenges of particular paleoproxies need to be recognized, while combining many types of data to estimate as much as possible about the past and being cognizant of the specific limitations of each proxy (Lomax and Fraser 2015).

How CT Is Related to LMA Components: Thickness and Density in Gymnosperms

LMA depends on leaf thickness and density (Niinemets 1999). Although leaf thickness and density have been found to be highly variable and can be independent from each other (Niinemets 1999; Veromann-Jürgenson et al. 2017), CT was related similarly to both components of LMA (fig. A2). Yet although the relationship between CT and leaf thickness, as well as leaf density, was significant, the correlations nevertheless were weak because of the high variability in all traits in this large sample. Since structural plasticity has been observed between leaf cohorts in conifers (e.g., LMA differences between growth light conditions or position in the canopy; Niinemets 2002, 2014; McGarvey et al. 2004), the CT versus LMA component relationships were modified by leaf shape, light, and sampling site (tables 1, 2). For example, CT and leaf thickness were highly correlated in full-light leaves from the Botanical Garden at the Natural History Museum of Denmark. However, in needles, the correlations between CT versus leaf thickness and density were not significant, demonstrating further that the LMA-CT relationship is not overarching across all gymnosperms. Although both leaf thickness and density are plastic in response to light conditions (Niinemets 1999, 2001), leaf thickness depended on CT in all light conditions for the species from the Botanical Garden at the Natural History Museum of Denmark (tables 1, 2; fig. 2). This could be promising for deep-time analyses in cycads, as leaf thickness and LMA can give additional separate information about plant functioning and ecological strategies (Niinemets 1999; Soh et al. 2017).

At the same time, although generally less variable than leaf thickness (Niinemets 1999) and considered a good proxy for leaf construction costs (Puglielli and Varone 2018), density correlated with CT only in scales, in full-light leaves in the sample from the Botanical Garden at the Natural History Museum of Denmark, and, likely because of the large sample, in species from the University of Tasmania (tables 1, 2). Because LMA has been demonstrated to be more tightly correlated with density than with leaf thickness in some gymnosperms (Veromann-Jürgenson et al. 2017), the weak CT versus density relationship should also be taken into account when employing the LMA-CT proxy.

Conclusions

Overall, our results indicate that CT and LMA do not have a strong relationship across our sample of gymnosperms, but the relationship depends significantly on leaf type, taxonomic affinity, and environmental conditions of the measurements’ location. Nevertheless, we suggest that CT has the potential to be used as a paleoproxy. Extrapolating the LES traits from relatively
abundant fossilized cuticular material is very appealing and could be used for inferring palaeoecological strategies for broad- and scale-leaved species. The reliability of the proxy increases as one moves toward the end of the LES, representing slow returns of investment of carbon and nutrients. While CT could as one moves toward the end of the LES, representing slow re-
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