# Manchester Metropolitan University

# A comparison of stomatal traits between contemporary and fossil leaves of *Melaleuca quinquenervia*: Do they reflect climate variation?

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## ARTICLE INFO

## ABSTRACT

Stomatal traits have been shown to vary in predictable ways in response to environmental change in many plant species. As a consequence, stomatal traits in fossil leaves are sometimes used as proxies for past $CO_2$ and climate. Here we investigate the influence of temperature, rainfall and $CO_2$ on stomatal traits in plant cuticle fine details of *Melaleuca quinquenervia*. We use both modern and fossil leaves deposited over the last c. 7500 years to evaluate the effect of $CO_2$, and modern leaves for climate variables. We found a significant negative relationship between stomatal size and density across both modern and fossil leaves of *M. quinquenervia*. However, we were unable to find any relationship between stomatal traits and $CO_2$ across a range from 260 to 380 ppm. We were unable to find any robust relationships between stomatal traits and either evaporation or temperature using the modern dataset. Apogeotropic roots account for the lack of stomatal anatomy response to evaporation in sites that experiences inundation. We conclude that stomatal size is a highly plastic trait in this species and changes do not necessarily reflect functional changes in the leaves.

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## 1. Introduction

Plants are capable of changing in response to a range of conditions. Physical changes can be over the life time of the plant, a plastic change. Plasticity maximizes survival in the face of environmental variables such as light, rainfall, humidity, temperature, $CO_2$ and nutrients. Plant plasticity manifests in a number of ways including changes in leaf size and shape, and stomatal traits that can often be measured in fossils. Stomatal trait changes in response to change in climate variables has been shown many times, initially in the seminal work of Woodward (1987). Woodward (1987) demonstrated a decrease in stomatal density in response to increasing atmospheric $CO_2$ concentrations. Low stomatal densities in a high $CO_2$ atmosphere reduce stomatal conductance in climates with high water vapor pressure deficits (Woodward, 1987). Stomatal changes sometimes influence the maximum potential uptake of $CO_2$ and control the rate of water loss in leaves. Maximum potential water loss through stomata ($g_{wmax}$) is a function of stomatal size and density, and is usually directly related to stomatal conductance for a range of plants. For example, Feild et al. (2011) analyzed 87 basal angiosperm species from across the world and found a strong, positive relationship between $g_{wmax}$ and measured stomatal conductance.

Maximum potential water loss through stomata determines the upper limit to stomatal conductance and therefore limits photosynthesis and whole canopy gas exchange (de Boer et al., 2011). It can vary in response to changing environmental conditions; for example, it has been shown to increase with decreasing $CO_2$ thereby increasing the capacity for $CO_2$ uptake as water availability declines for one lycophyte, one fern and two angiosperms (Franks et al., 2012).

Stomatal size and density are affected by environment and climate factors other than $CO_2$ and sometimes exclusive of $CO_2$. These include water availability (Fraser et al., 2009), temperature (Beerling and Chaloner, 1993), nutrients (Peñuelas and Matamala, 1990), light (Onwueme and Johnston, 2000), soil salinity (Bray and Reid, 2002) and humidity (Nejad and Van Meeteren, 2005). It is important to note that stomata do not always alter stomatal traits in response to climate and environment factors; these species may have a higher capacity to alter stomatal conductance (Haworth et al., 2015). However, stomata from plants that do have varying anatomy as forced by climate and environment change, are often used as proxies to infer past climate.

There have been times in the past when the atmospheric $CO_2$ concentration has been relatively stable. One of these periods was the Holocene epoch when $CO_2$ changed by less than 20 ppm over the 11,700 years prior to ~1850 AD (Indermühle et al., 1999). Therefore, changes in stomatal traits over this time may be due to other environmental factors, such as temperature or rainfall variation. Considerable

global and regional scale climatic variability occurred through the Holocene (Wanner et al., 2011). For example, the northern Australasian monsoon intensified between 7500 and 5000 years before present (BP), resulting in high rainfall, at times above that of the modern range (Shulmeister and Lees, 1995). In a review of eastern Australian Holocene rainfall varability, Reeves et al. (2013) found that the majority of studies indicate a decline in effective precipitation after 5000 BP. These rainfall fluctuations may have influenced stomatal traits through the Holocene.

The relative stability of atmospheric $CO_2$ during the Holocene provides an opportunity to remove this variable from possible influencers on stomatal anatomy. Thus, we examine how stomatal traits may have varied over this time in response to temperature and class A pan evaporation (hereafter, evaporation). In this study, we investigated responses of *Melaleuca quinquenervia* (Cav.) ST Blake to a range of environmental variables. Leaves of *M. quinquenervia* obtained from both lake sediments with ages up to 7.3 ky (hereafter referred to as fossils) and modern leaf litter (hereafter referred to as the modern dataset) are tested. *Melaleuca quinquenervia* has been chosen as the study species as it is abundant in the lake sediment and presented an opportunity to study the effect of environmental variables on stomatal anatomy of an Australian native tree across 7.3 ky.

Many studies have been conducted that are similar to this one, for example, buried leaf material of *Betula pendula* spanning 50 years has been regressed against $CO_2$ change with a very strong correlation demonstrated (Wagner et al., 1996). An Australian example of similar studies has been conducted on *Eremophila deserti* leaf material preserved in amberat from middens of *Leporillus conditor* and *L. apicalis* (stick nest rats, Atchison et al., 2000). This material was up to 7500 years old and compared with extant leaf collections and herbarium specimens, Atchison et al. (2000) discussed the likelihood of El Niño Southern Oscillation (ENSO) and the Australian monsoon causing precipitation regime changes leading to stomatal index adaptation. This study is an analysis of the correlations of stomatal measurements to temperature and rainfall parameters.

## 2. Methods

### 2.1. Study site and species

*Melaleuca quinquenervia* is an Australian native tree species that occurs on floodplains, in wetlands (Greenway, 1994), and the littoral zones of lakes (Lockhart et al., 1999). When inundated, the tree grows roots from epicormic buds in an upward direction thus allowing oxygen uptake into roots (McJannet, 2008). It is categorized as scleromorphic, based on features such as fibrous leaves with a thick cuticle, and an evergreen habit (Hill, 1998). The habit is open, as the leaves are amphistomatic (Jordan et al., 2014). Stomata are evenly distributed across the leaf surface (Fig. 2). *Melaleuca quinquenervia* occurs along the east coast of Australia in Queensland and New South Wales (Brophy et al., 2013; Tibby et al., 2016). It also occurs naturally in Indonesia, Papua New Guinea, and New Caledonia (Brophy et al., 2013), the Hawaiian Islands and has naturalized in southern Florida where it is an invasive species (Pratt et al., 2014).

We measured stomatal traits from *M. quinquenervia* leaves collected between 1992 and 2003 from Carbrook Wetland (27.7°S, 153.2°E), a seasonally inundated wetland approximately 40 km south of Brisbane (Fig. 1; Greenway, 1994). We also measured stomatal traits of fossil leaves obtained from a sediment core taken from Swallow Lagoon on North Stradbroke Island, Queensland, Australia (27.5°S, 153.4°E). Swallow Lagoon is a small (0.27 ha), oligotrophic, freshwater lake located high in the dunes of North Stradbroke Island, the world's second largest sand island. The lake is perched, meaning that it is separated from the regional water table. Variation in water depth is therefore a function of the balance between precipitation and evaporation. Extant
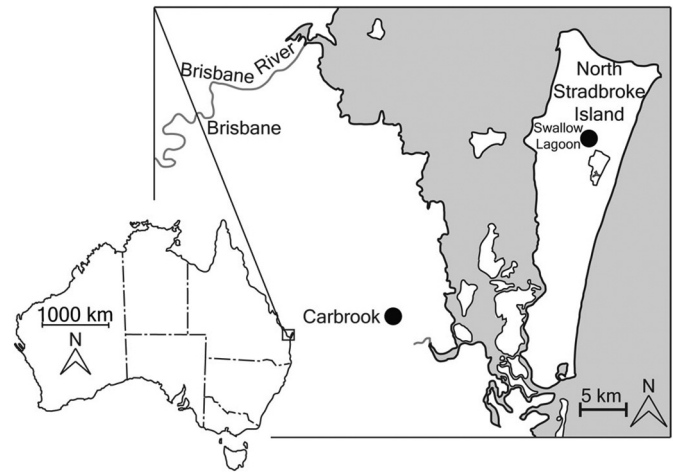


Fig. 1. Location of field sites where the modern leaves (Carbrook wetlands) and fossil leaves (Swallow Lagoon) of *M. quinquenervia* were collected (map modified from fig. 1c of Tibby et al., 2016).

populations of *M. quinquenervia* continue to grow as fringing vegetation around the lake.

As the modern specimens all came from Carbrook Wetland and all fossil specimens came from Swallow Lagoon, we assume that nutrient availability and soil salinity were similar for all modern and all fossil leaves collected for this study. We also assume that light availability did not affect leaves as the plants have open canopies and do not have significant self-shading.

### 2.2. Modern leaves

Modern *M. quinquenervia* leaves have been taken from samples collected for a leaf-litter monitoring project, the initial results of which are reported in Greenway (1994). Leaf litter was collected in a litter trap at four-week intervals, between April, 1992 and July, 2003, from Carbrook Wetland (Fig. 1). For our study, one leaf has been sampled every second month, beginning in April 1992 with an even spread of sampling conducted over the 12 year time frame (n = 4, 5 or 6 per year). A total of 61 leaves have been analyzed.

### 2.3. Fossil samples

Sediment cores were taken from a platform, anchored in the deepest part of Swallow Lagoon, in March, 2011. The record is a composite of two cores. Core SL1 (150–375 cm), collected using a Livingstone corer, has been extruded in the field where it was sealed and stored for transportation back to the laboratory for analysis. Core SLP3 (0–250 cm) was collected using a clear perspex soft sediment piston corer. This was extruded in the field at 1 cm increments from 0 to 150 cm, and the remainder as a single section which was sectioned in the laboratory. The two cores were correlated stratigraphically, using a distinct 5 cm thick band of sand evident in both cores at 220 cm.

The cores were sampled at contiguous 1 cm intervals and the sediment was washed through a 500 μm sieve to obtain macrofossil remains. The retained fraction was rinsed in distilled water and *M. quinquenervia* leaf fragments were selected for analysis. Fragments of *M. quinquenervia* were identified using distinctive morphological features, such as thick veins, epidermal color and anatomy of the spongy mesophyll. A chronology for the core, based on 12 ^{14}C dates on terrestrial leaf macrofossils, indicates the sediment covers the last 7300 years (Barr et al., 2019; Tibby et al., 2016). The distribution of leaves varied through the sediment column and specimens were collected where samples of sufficient size and number were available. A total of 93 sediment levels have been analyzed.
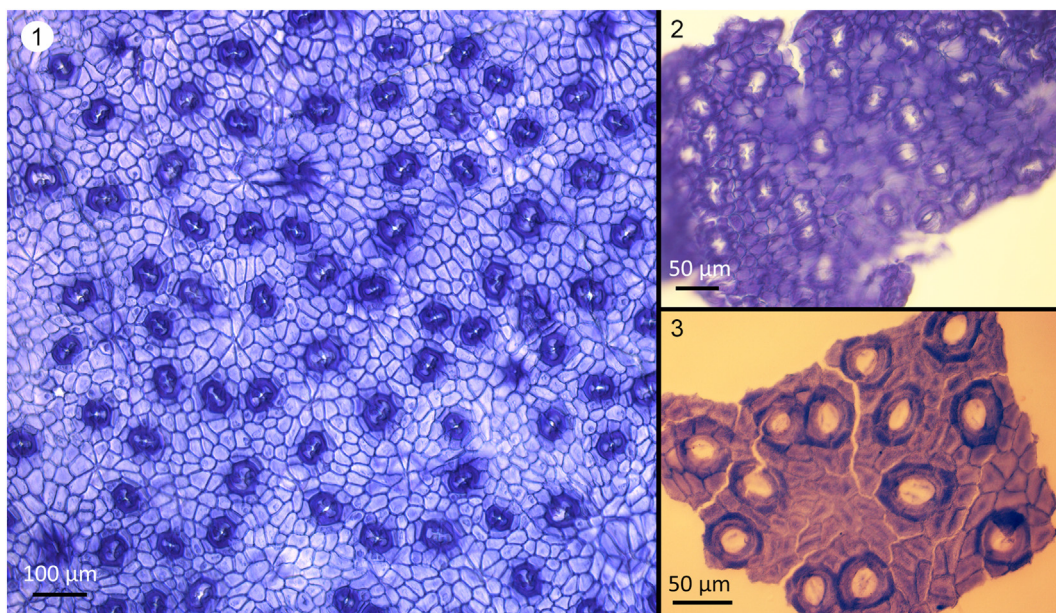
**Fig. 2.** Fossil and extant leaf cuticle of *Melaleuca quinquenervia*. (1) extant *M. quinquinervia*, scale = 100 μm; (2) fossil leaf cuticle collected from a depth of 173 cm, scale = 50 μm; (3) fossil leaf cuticle collected from a depth of 154 cm, scale = 50 μm.

## 2.4. Cuticle preparation

### 2.4.1. Modern leaves

Cuticle preparation of modern leaves followed methods described in Hill et al. (2015). Briefly, single, 1 cm² pieces were cut from each leaf margin half way along the lamina. These were placed in separate test tubes, covered in 80% ethanol *v/v*, and left for 24 h at room temperature. The ethanol was then removed and leaves covered in a 2:1 solution of 35% hydrogen peroxide and 80% ethanol (*v/v*). The test tubes were then placed in a water bath in a fume cupboard and gently heated until the leaf samples were translucent, indicating that the cuticle had separated from the rest of the leaf. The leaf samples were gently rinsed with reverse osmosis (RO) water, and debris was brushed away from the cuticle with a fine camel hair brush. The cuticles were stained with 0.05% crystal violet (*w/v*) for 10 s. Leaf cuticles were transferred to a slide, and mounted in warm phenol glycerine jelly. A coverslip was placed on top of the sample and left overnight at room temperature. Nail polish was then applied to the coverslip edges to preserve the cuticles from dehydration.

### 2.4.2. Fossil samples

Leaf fragments were covered in a solution of 10% aqueous chromium trioxide (*w/v*), and left for between 1 and 5 days at room temperature. The resulting leaf cuticles were rinsed in reverse osmosis (RO) water, then stained and then mounted onto slides using the same method as described for the modern leaves. Fossil cuticle slides are stored in The University of Adelaide collection.

## 2.5. Stomatal measurements

Leaf cuticles were examined with a light microscope (Olympus AX70), and photomicrographs were taken with an Olympus UC50 camera. Micrographs were analyzed using imaging software (AnalySIS version 6.0.6001 Service Pack 1 Build 6001, Acer, Australia). In the case of the fossil leaves, three pieces of leaf cuticle per one cm of leaf core section were measured; these pieces of leaf cuticle were often fragmentary. Stomatal size (μm²) was calculated as the mean of length by width of five guard cell pairs per piece of cuticle. Stomatal density (stomata mm⁻²) was determined by counting the number of stomata in a
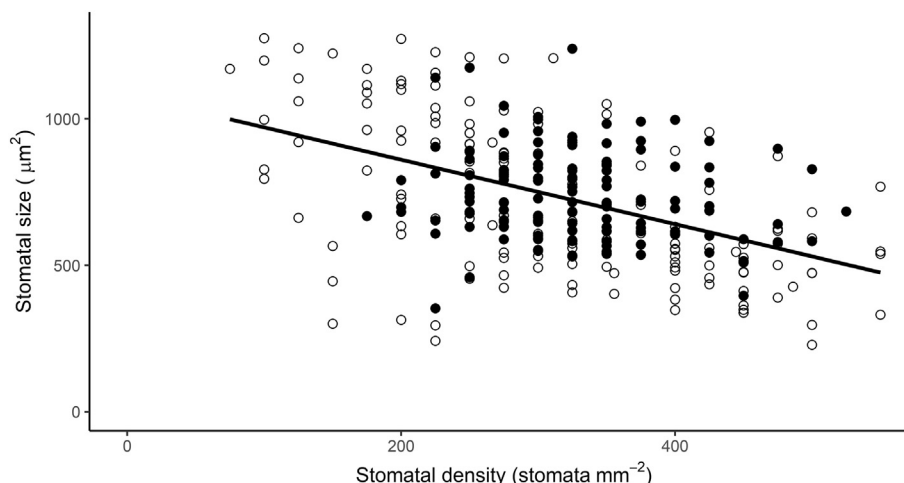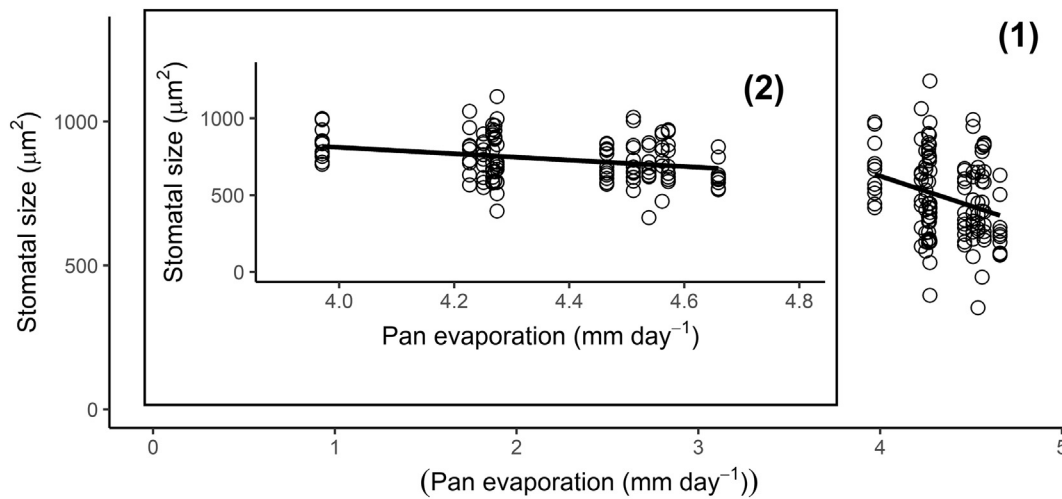


**Fig. 3.** The relationship between stomatal density and size for modern (closed circles) and fossil (open circles) leaves of *M. quinquenervia*. The line is a linear model.
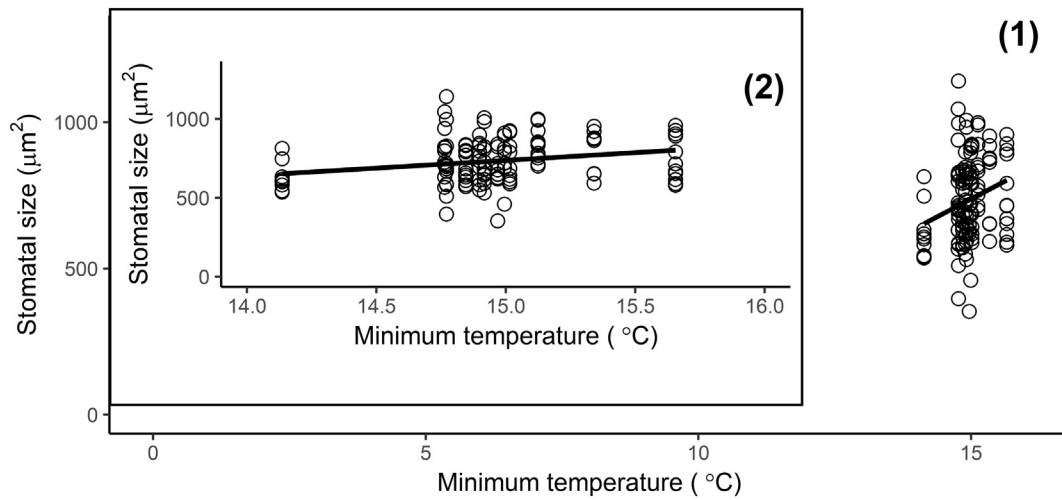
**Fig. 4.** Relationship between mean daily minimum temperature and stomatal size in modern *M. quinquenervia* leaves. A linear model is also shown ($r^2 = 0.051$, P = .007). (1) is a graph with an x-axis starting at an origin of zero, (2) is a graph with an x-axis origin starting at 14 °C to illustrate the relationship clearly.



**Fig. 5.** Relationship between Class A pan evaporation and stomatal size in modern *M. quinquenervia* leaves. A linear model is also shown ($r^2 = 0.071$, P = .002). (1) Is a graph with an x-axis starting at an origin of zero, (2) is a graph with an x-axis origin starting at 3.9 mm day$^{-1}$ to illustrate a the relationship clearly.
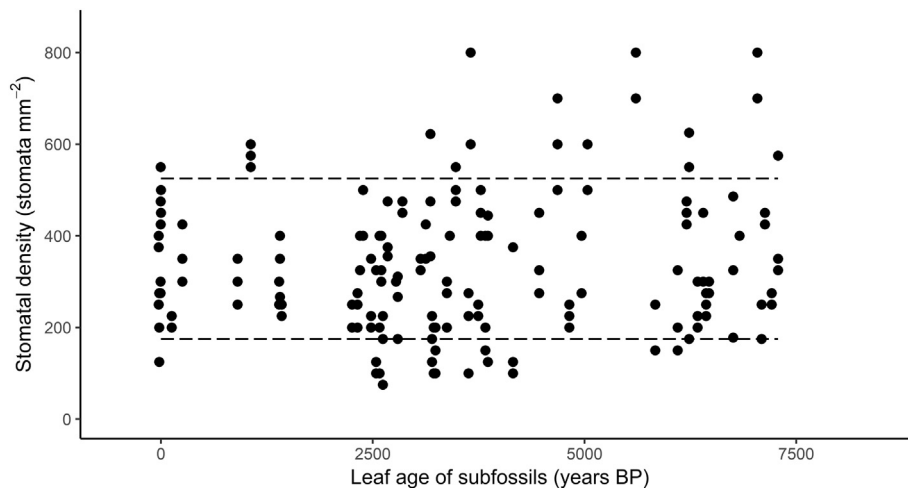


**Fig. 6.** Changes in stomatal density during the Holocene for *M. quinquenervia* from fossil samples. The dotted lines indicate the maximum and minimum stomatal density of the modern dataset. 62 leaf cuticles have been used for this plot and each stomatal density replicate is plotted as a single data point, 177 points in total are included on the figure.
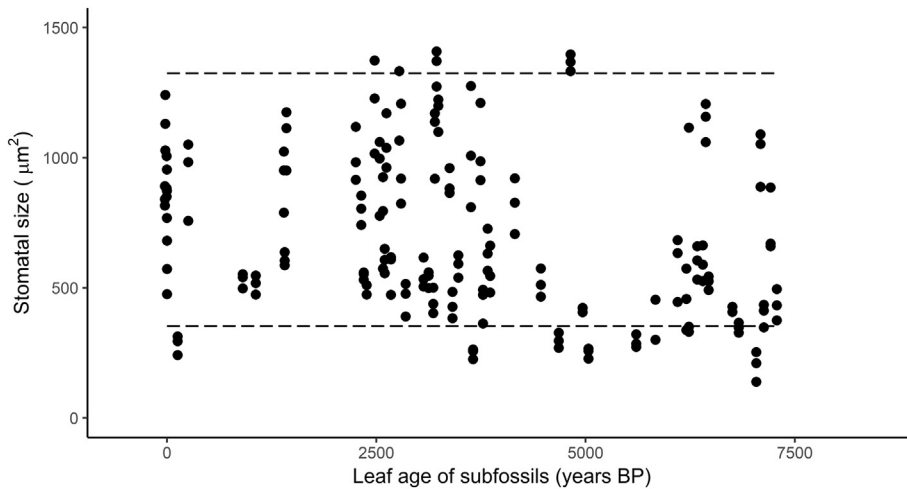
**Fig. 7.** Changes in stomatal size during the Holocene for *M. quinquenervia* from fossil samples. The dotted lines indicate the maximum and minimum stomatal size of the modern dataset. 93 leaf cuticles have been used for this plot and each stomatal size replicate is plotted as a singl data point, 186 stomatal size measurements are included.

minimum 100 × 100 μm area on each cuticle. Where possible, a 200 × 200 μm area was used for stomatal counts with three of these areas counted per cuticle. Stomata have been counted according to protocol by Poole and Kürschner (1999). Three of the one cm core sections had only one leaf fragment available for counting density, and three had only two leaf fragments available for this purpose. Final numbers for stomatal density were n = 62, and for stomatal size were n = 93. There were fewer measurements of stomatal density as some leaf fragments were too small to determine stomatal density. The leaf cuticle fragments were also often too small for epidermal cell counts, thus stomatal index claculations are not viable for this particular material.

For the modern leaves we present individual measurements of the abaxial and adaxial leaf surfaces for stomatal density and size and compare these with stomatal traits from fossil samples. This has been done as we cannot be sure as to whether the fossil leaf cuticle fragment was adaxial or abaxial. Thus, all possible measurements have been taken and plotted individually to remove any smoothing likely to occur from presenting means.

### 2.6. Climate data

Environmental data were obtained using the Scientific Information for Land Owners (SILO) database for the location 27.7°S, 153.2°E. SILO is a database compiled and interpolated from observational data collected by the Australian Bureau of Meteorology (Jeffrey et al., 2001) The data obtained from SILO were mean annual values for minimum air temperature (°C), and class A pan evaporation (mm year$^{-1}$) (Table 1).

The mean annual minimum temperature ranged from 14.1 °C in 1994 dataset to 15.7 °C in 1998. Class A pan evaporation ranged from 3.97 mm year$^{-1}$ in 1999 to 4.66 mm year$^{-1}$ in 1994. As leaf longevity for *M. quinquenervia* is 2–4 years (M. Greenway, Griffith University, Australia pers. comm; Van et al., 2002), climate data from two years prior to leaf litter collection have been compared with stomatal data.

### 2.7. Statistical analyses

All statistical analyses have been run in RStudio (Team, 2019). Data have been read into RStudio using readr (Wickham et al., 2018). The package psych (Revelle, 2018) has been used to create a PCA to confirm use of climate variables in models. Outliers have been removed using the Outlier removal by the Tukey rules on quartiles ± 1.5 IQR R script (Dhana, 2017). Linear models have been used to investigate the relationships between stomatal density and size for both modern and fossil specimens, RStudio package gvlma (Pena and Slate, 2019) has been

used for this purpose as well as testing for validity of linear model assumptions. These models have been run using ggplot2 (Wickham, 2016). Graphs have been plotted using the R packages plotrix (Lemon, 2006), viridis (Garnier, 2018) and cowplot (Wilke, 2019). ANOVAs were performed to test significance between the measures of fossil and modern populations of stomatal size and density.

## 3. Results

### 3.1. Stomatal size and density

Outliers have been removed from the stomatal size dataset as some values were deemed as outliers. Following this removal, the assumptions for a linear model was confirmed as valid. The differences between fossil and modern stomatal density and size were not statistically significant. The difference between modern and fossil stomatal density is not statistically significant with a P-value = .264; the P-value = .327 for the difference between modern and fossil stomatal size. The fossil leaves contained the largest and smallest stomata, and likewise the highest and lowest stomatal densities (Table 2). For the combined modern and fossil datasets, there was a negative relationship between stomatal size and density (Fig. 3). Notably, there was a greater range in values for stomatal size and density in the fossil dataset than the modern one (Figs. 6 and 7).

### 3.2. Correlation of stomatal size to climate

Stomatal size measurements correlated with both minimum temperatures and evaporation. There were also no linear correlations

**Table 1**
Abbreviations, associated full terms and descriptions used in this paper.

| Abbreviation | Terminology | Description |
|---|---|---|
| CO$_2$ | Carbon dioxide | Atmospheric concentration of CO$_2$ |
| ENSO | El Niño Southern Oscillation | The oscillation between warming of the central and eastern tropical Pacific Ocean and cooling of the same region (Bureau of Meteorology, 2008) |
| Stomatal density | Stomatal density (stomata mm$^{-2}$) | Measurement of the number of stomata per square millimetre of leaf cuticle |
| Stomatal size | Stomatal size (μm$^2$) | Calculation of length by width of stomata, presented as the mean of five stomata per image |
| ANOVA | Analysis Of VAriance | Statistical analysis of variation within and between groups |
| P | P-value | Probability that the hypothesis is true |

**Table 2**

Ranges and means of stomatal parameters for modern and fossil datasets of *Melaleuca quinquenervia*. For the modern dataset, both stomatal size and density have the same number of observations, n = 126. For the fossil material stomatal density measurements, n = 177, for the fossil material stomatal size measurements n = 184.

| | Modern dataset | | | Fossil dataset | | |
|---|---|---|---|---|---|---|
| | Min. | Max. | Mean ± SE | Min. | Max. | Mean ± SE |
| Stomatal density (stomata mm$^{-2}$) | 175 | 525 | 327 ± 6 | 75 | 900 | 344 ± 12 |
| Stomatal size (μm$^2$) | 353 | 1240 | 740 ± 13 | 139 | 1699 | 710 ± 24 |

between stomatal size or density from the modern dataset with mean daily minimum temperature (Fig. 4). There is a significant, weak, correlation between minimum temperature and stomatal size (P = .007); variation in minimum temperature explained 5.1% of the variation in stomatal size ($r^2 = 0.051$ for the correlation between minimum temperature and stomatal size). The relationship is positive, thus smaller stomata formed during periods of cooler minimum temperatures and stomata are larger during warmer minimum temperatures. Stomatal size of leaves from the modern dataset was significantly, though weakly, correlated with evaporation, P-value = .002 (Fig. 5). Variation in evaporation explained 7.1% of the variation in stomatal size for *M. quinquenervia* ($r^2 = 0.071$ for the correlation between evaporation and stomatal size). The relationship is negative whereby small stomata are formed during periods of lower evaporation and larger during periods of higher evaporation. By contrast, stomatal density did not correlate with evaporation.

## 4. Discussion

This study investigated relationships between stomatal morphology and temperature and evaporation using *M. quinquenervia* leaf litter collected over 12 years from 1992 to 2003. We also measured stomatal traits of fossils of the same species collected from a lake covering the period from 7300 years ago to 1975 CE.

### 4.1. Stomatal density and size

The negative relationship we observed between stomatal density and size of both the modern and fossil *Melaleuca quinquenervia* leaves was similar to observations reported in previous studies on other species. These studies have shown a negative, logarithmic relationship between stomatal size and density (e.g., Brodribb et al., 2013; Franks and Beerling, 2009; Haworth et al., 2018). This relationship between stomatal size and density is likely to be due to the limited leaf area that is available for stomata (Brodribb et al., 2013), but it also has functional significance as more, smaller stomata result in a higher maximum potential water loss than fewer, larger stomata with deeper pores (Brodribb et al., 2013; Franks and Beerling, 2009). It is important to note that these relationships hold for the same or closely related species, not among diverse groups.

### 4.2. Climate–stomata correlations

Stomatal size hardly varied with short-term changes in minimum temperatures in the modern *M. quinquenervia* leaves (Fig. 4). A larger stoma creates a longer diffusion path for water to travel along when exiting the leaf during transpiration (Nobel, 2009), and thus reduces transpiration. We expected to see that larger stomata in *M. quinquenervia* leaves formed during periods of warmer minimum temperatures. Temperature is directly proportional to vapor pressure deficit (VPD), and warmer minimum temperatures create a larger VPD and thus a greater driving force for water to evaporate through open stomata. For a given VPD, larger stomata would slow evaporation relative to smaller stomata, and thus retain water for longer. However, due to the short time frame for possible leaf acclimation, or alternatively the incapacity for stomatal size to acclimate to change in temperature,

resulted in statistically weak variation in stomatal size as minimum temperatures varied.

There was no correlation between stomatal density and temperature, thus, it could be argued that there was no functional change in potential water loss as only stomatal size changed weakly in response to temperature. The lack of response by stomatal density to temperature leads suggests that stomatal size is a more plastic phenotype than the former two variables with temperature changes.

There is statistically weak correlation between stomatal size and evaporation. It is possible, however, that stomatal size in leaves of *M. quinquenervia* is only weakly correlated with evaporation because it frequently occurs in areas where the water table is high (e.g. wetlands and lake edges). It also has apogeotropic roots that are adapted to rising and falling water tables. McJannet (2008) demonstrated that stand transpiration of *M. quinquenervia* was unaffected by variation in water table depth due to these root systems. Thus, in this high water environment, evaporation is unlikely to be a limiting factor.

In the 7300 year Holocene leaf accumulation, the stomatal size of the fossil samples regularly fall outside the range of that of the modern dataset. The correlation of stomatal size of the modern dataset to minimum temperature likely reflects the correlation between these variables during the Holocene, although it is important to note the temporal range between these two is quite different, since we are comparing a 12 year dataset with a 7300 year one and the range of temperatures and evaporation will be different, but we do not know the extent of this. The use of stomatal features as paleo-environmental indicators may be constrained by natural variability when modern relationships are produced under field conditions. As such, the weak correlation between stomatal size and minimum temperature cannot be considered for use as a paleo-temperature proxy. Change in stomatal anatomy has been shown to correlate with $CO_2$ changes, though we do not consider this to be the case for change in *M. quinquenervia* stomatal size as $CO_2$ only increased by ~20 ppm during the Holocene (Indermühle et al., 1999).

During the Holocene, large changes in El Niño-Southern Oscillation (ENSO) caused variability in rainfall events. In particular, an intensification of ENSO in the late-Holocene has been noted in a range of proxies from the Eastern Pacific (e.g., Conroy et al., 2008; Koutavas and Joanides, 2012; Moy et al., 2002) and Australia, including from the Swallow Lagoon study site (e.g., Barr et al., 2019; Donders et al., 2007; Quigley et al., 2010; Shulmeister and Lees, 1995). However, an intensification of ENSO leading to drought events mediated by *M. quinquenervia*'s root system is thus not reflected in stomatal size during the Holocene.

Barr et al. (2019) used sediments from the same samples in the same region of Australia to estimate a rainfall proxy for Stradbroke Island during the Holocene. These authors used carbon isotope values for this reconstruction and our data are not similar to theirs (Barr et al., 2019). We were unable to demonstrate a rainfall response in stomatal density or size and thus could not reconstruct a proxy for rainfall. Potential reasons for this include stomatal size and density responses occuring at a longer time scale-years than carbon isotopes-weeks. It is also possible that *M. quinquenervia* stomata are not sensitive to environmental changes, whereas carbon isotope composition is sensitive to rainfall variation. Finally, it is always going to be more difficult to obtain stomatal data because of the limited amount of fossil material available, whereas the *M. quinquenervia* carbon isotope data have been obtained

before the sampling for stomata was undertaken. Thus, that carbon isotope composition may be a better rainfall proxy than stomata.

However, more evidence of temperature and evaporation forcing on stomatal morphology is required before we can use these data to create proxies of Holocene climate. The relationships found here are weak for temperature and evaporation response and more data are needed to detect subtle correlations with climate. These include collection of herbarium data from a wider spatial and temporal range, including invasive *M. quinquenervia* from Florida and an increase in the sample size of fossil leaves.

## 4.3. Conclusion

This study showed that stomatal size correlated weakly with minimum temperatures suggesting a response by *Melaleuca quinquenervia* leaf anatomy to changes in VPD. Apogeotropic roots account for the lack of stomatal anatomy correlation to evaporation. The fossil dataset indicates that there may have been climate influences forcing stomatal change, however, stomatal size and density are not reliable proxies of paleo-environments. Analysis of a larger dataset of fossil and modern leaves is required to detect any more subtle correlations between stomatal anatomy and climate variables.

## Acknowledgements

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