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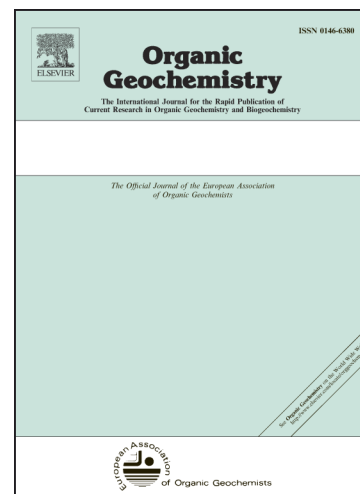
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Variation in leaf wax *n*-alkane characteristics with climate in the broad-leaved paperbark (*Melaleuca quinquenervia*)

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ABSTRACT

In higher plants, leaf waxes provide a barrier to non-stomatal water loss, and their composition varies both between and within species.

Characteristics of *n*-alkanes, a suite of ubiquitous compounds in these waxes, are thought to be influenced by the availability of water and the temperature in a plant's growing environment. Longer *n*-alkane distributions with less variability in chain length are hypothesised to confer greater resistance to non-stomatal water loss and thus are expected in higher abundance in desiccating environments. Relationships between the distribution of *n*-alkane characteristics and both precipitation and temperature have previously been observed. Despite this, it is unclear whether *n*-alkane chain length distributions vary plastically in response to climate, or whether they are fixed within populations in different climate settings. To better understand this, we examine the relationship between *n*-alkane characteristics of *Melaleuca quinquenervia* and both spatial and temporal climate variation. Across eastern Australia, we find that *n*-alkane homolog concentrations and distributions in leaves of *M. quinquenervia* do not vary with climate where samples are proximate, even when climate shows significant variability. However, the concentration and distribution of

n-alkane homologs do differ considerably between geographically separated populations in very different climate regimes. These results suggest *n*-alkane characteristics are not a plastic response to climate variability, and instead are likely fixed and could be driven by genetic differences between populations. This has important implications for the use of *n*-alkane characteristics as palaeoenvironmental proxies.

Keywords: Leaf wax *n*-alkanes; chain length distribution; precipitation; temperature; *Melaleuca quinquenervia*

1. Introduction

The leaf waxes of higher plants contain a mixture of long chain aliphatic *n*-alkyl derivatives (e.g., *n*-alkanes, *n*-alkanols, *n*-alkanoic acids), triterpenoids (Schuster et al., 2016) and minor secondary metabolites (Eglinton et al., 1962; Eglinton and Hamilton, 1967; Kunst and Samuels, 2003; Jetter et al., 2006; Schuster et al., 2016). Long chain *n*-alkanes ($n\text{-C}_{25}$ – $n\text{-C}_{35}$) are a ubiquitous component of leaf waxes, with their distribution in leaf wax typically displaying odd over even predominance of homologs with maxima at $n\text{-C}_{27}$, $n\text{-C}_{29}$ or $n\text{-C}_{31}$ (Kunst and Samuels, 2003). A key function of leaf waxes is to reduce water loss through the cuticle (Riederer and Schreiber, 2001; Jetter and Riederer, 2016), with other roles including the reduction of solute leaching from inside cells and protection against UV-radiation damage (Koch and Ensikat, 2008). The prevailing mechanism for

how leaf waxes reduce water loss through the cuticle is explained by the barrier membrane model (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994; Riederer and Schreiber, 1995; Jetter and Riederer, 2016). In this model, leaf waxes form micro-crystalline (crystallite) and unstructured (amorphous) zones, with alignment of hydrocarbon (i.e. *n*-alkane) backbones in crystallites creating impermeable zones in the leaf cuticle (Riederer and Schreiber, 1995; Koch and Ensikat, 2008).

It is hypothesised that the impermeability of the crystallites forces water to travel around them through a more amorphous matrix created by alicyclic compounds. This creates a lengthened transport pathway that increases the resistance of the cuticle to water passing through it (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994; Riederer and Schreiber, 1995; Jetter and Riederer, 2016). A prediction of this model is that greater resistance to water loss is associated with greater concentrations of long-chain *n*-alkanes, as well as longer and more narrowly distributed *n*-alkane homologs i.e. low variability in constituent chain lengths. This would be advantageous to plants living in arid conditions (Riederer and Schneider, 1990; Riederer and Schreiber, 1995; Dodd et al., 1998; Dodd and Afzal-Rafii, 2000; Dodd and Poveda, 2003; Shepherd and Griffiths, 2006; Koch and Ensikat, 2008).

Modern calibration studies of *n*-alkanes in the context of climate from plants, soils and sediment have shown correlations between *n*-alkane characteristics and aridity (e.g., Vogts et al., 2009; Hoffman et al., 2013;

Leider et al., 2013; Tipple and Pagani, 2013; Carr et al., 2014; Bush and McInerney, 2015). Moreover, major climate events in the past are associated with large shifts in *n*-alkane chain length distributions (e.g., Smith et al., 2007; Baczynski et al., 2016). Together, observations of modern and ancient systems demonstrate potential for *n*-alkanes preserved in the geological record to hold palaeoclimate information (Smith et al., 2007; Bush and McInerney, 2013; Hoffman et al., 2013; Leider et al., 2013; Carr et al., 2014; Bush and McInerney, 2015; Baczynski et al., 2016; Diefendorf and Freimuth, 2017). Even so, the interpretation of bulk plant wax *n*-alkane signatures in the geological record using modern climate calibrations is not straightforward.

Many modern climate calibration studies of *n*-alkanes in plants and surface sediments rely on 'space-for-time' substitution where variations in space are used to infer variations in time. This approach limits the ability to perceive the responsiveness of the proxy to environmental change in modern systems (Pickett, 1989; Diefendorf and Freimuth, 2017). This is compounded in many cases by a lack of control on plant community turnover (e.g., Vogts et al., 2009; Hoffman et al., 2013; Carr et al., 2014; Bush and McInerney, 2015), with intrinsic differences in *n*-alkane production by different taxa having the potential to bias calibrations of *n*-alkane response to climate (Vogts et al., 2009; Diefendorf et al., 2011, 2015; Freeman and Pancost, 2014; Garcin et al., 2014; Diefendorf and Freimuth, 2017; Jansen and Wiesenberg, 2017; Howard et al., 2018). Complexities in plant wax *n*-alkane

inputs to sediments are also inherent, with most sedimentary records likely representing a complex mixture of regionally and locally sourced leaf wax *n*-alkanes (Schefuss et al., 2003; Diefendorf et al., 2011; Freeman and Pancost, 2014; Garcin et al., 2014; Rouillard et al., 2016; Diefendorf and Freimuth, 2017; Jansen and Wiesenberg, 2017; Howard et al., 2018). This complexity and the biases imposed suggests a need for careful interpretation of bulk sedimentary leaf wax *n*-alkane records (Diefendorf et al., 2011; Rouillard et al., 2016; Diefendorf and Freimuth, 2017; Jansen and Wiesenberg, 2017; Howard et al., 2018).

These complexities can be minimised through the application of single-species calibrations to single-species leaf wax *n*-alkane records. Such single-species records are rare, but Holocene sub-fossil leaves of the species *Melaleuca quinquenervia* (Cav. S.T. Blake) are well preserved in lake sediments from south-east Queensland, Australia (Tibby et al., 2016; Barr et al., 2019). These types of records present an opportunity to better use plant wax *n*-alkanes to understand Holocene climate in a region that is particularly sensitive to changes in the El Niño–Southern Oscillation.

There is uncertainty, however, in whether relationships between leaf wax *n*-alkane characteristics and climate in a single species reflect plastic responses to short-term climate variability or whether they reflect potentially genetically fixed features of plants in different ambient climate conditions (Diefendorf et al., 2015; Bender et al., 2017). There is evidence to suggest that genetics plays a large role in *n*-alkane biosynthesis, and that

there is potential for ecotypes of species, in terms of their *n*-alkane characteristics, to emerge where populations are geographically and climatically distinct (Schreiber and Riederer, 1996; Dodd et al., 1998; Dodd and Afzal-Rafii, 2000; Shepherd and Griffiths, 2006; Rajčević et al., 2014; Diefendorf et al., 2015). Confidently interpreting palaeodimate from changes in *n*-alkane characteristics of sub-fossil leaves requires a better understanding of modern *n*-alkane characteristics in relation to climate.

In this study, we explore variation in *n*-alkane characteristics with climate of *M. quinquenervia* through time in one place as well as on large and small spatial climatic transects. We aim to provide unique insights into the responsiveness of *n*-alkane characteristics to climate within a single species, thereby removing the effects of *n*-alkane production differences between plant groups, functional types and species. If *n*-alkane characteristics reflect a plastic response to short-term climate variability, then we expect to see significant correlation between climate and *n*-alkane characteristics of *M. quinquenervia* at all scales, both temporally and spatially. If, however, *n*-alkane characteristics reflect genetically fixed features of ecotypes living in different ambient climates, we expect to observe distinct *n*-alkane characteristics in geographically and climatically distinct populations of *M. quinquenervia*. We measure leaf wax *n*-alkane characteristics of *M. quinquenervia* across variable precipitation and temperature conditions in three different sampling sets of living plants: (1) a time-series of 11 years at a single site, (2) a south-east Queensland (SEQ)

transect across ~150 km and (3) a cross-Queensland (QLD) sample set comparing SEQ with Cape York, that are separated by ~1500 km (Fig. 1).

2. Material and methods

2.1. Study species

Melaleuca quinquenervia, the broad-leaved paperbark or five-veined paperbark, is an evergreen tree of the family Myrtaceae that typically ranges in height in mature trees from 8 to 12 m, though smaller or larger trees are not uncommon (4–25 m) (Boland et al., 2006). This species inhabits coastal areas and is associated with wetlands, with its native geographic range extending from southern New South Wales to Cape York within Australia and continuing into southern Papua New Guinea and New Caledonia. The species is naturalised in other regions, notably the southern United States of America (Ireland et al., 2002). In Australia, new leaf growth in this species begins in mid-winter and continues through to early summer (Serbesoff-King, 2003). The growing season for this species is therefore considered as the Austral winter and spring (June to November, inclusive).

2.2. Study sites and sampling

2.2.1. Time-series study

Long term sampling (11 years) for our time-series study was undertaken via a litter trap approach at Carbrook Wetlands (27.690°S,

153.276°E), a part of the Native Dog Creek catchment and the Logan River floodplain (Fig. 1). Approximately 164 ha of the wetland is comprised of *M. quinquenervia* forest (Greenway, 1994; Tibby et al., 2016). The sample collection area for our time-series study had a tree density of 2175 trees per ha, with a mean tree height of 18.6 m (± 4 m, 1 standard deviation). The mean tree diameter at the site was 17.8 cm (± 9 cm, 1 standard deviation). The tree density and the presence of leaves from species not overhanging the litter traps suggests leaves were integrated from many individuals in the sampling area. Litterfall was collected in a raised 0.25 m² (0.5 m \times 0.5 m) tray in one area of Carbrook Wetlands, with leaf litter sampled approximately every four weeks between April 1992 and July 2003 (Tibby et al., 2016). Leaf litter samples from 45 collection periods were used in this study, with a temporal resolution of approximately three months. Nine leaves from each of the collection periods were selected and homogenised under liquid nitrogen in a ceramic mortar and pestle.

2.2.2. South-east Queensland (SEQ) study

The SEQ study area is located in a humid subtropical climate zone (Fig. 1), as defined by the modified Köppen-Geiger climate classification (Peel et al., 2007). Sampling was undertaken at 21 sites across SEQ, with a cluster of small leaves (~15–20) from near the growing tip, assumed to be relatively young leaves, taken from one tree at each sampling site at a height of 1–2 m using pruning shears. Leaves were consistently taken from

the northern side of trees in open sun to mitigate the influence of sun versus shade leaves (Suh and Diefendorf, 2018). From the cluster of leaves sampled, the nine smallest leaves were selected for homogenisation. After collection, leaves were stored in paper bags and subsequently oven dried at 50 °C for 48 h, prior to homogenisation under liquid nitrogen in a ceramic mortar and pestle.

2.2.3. Cross-Queensland (QLD) study

The QLD study area encompasses the SEQ study area, along with four other sites on the Cape York Peninsula in far-north Queensland, located in a tropical savanna climate zone (Peel et al., 2007) and approximately 1500 km from the SEQ sites (Fig. 1). These sites were chosen to be sampled because of their distinct climate compared to the SEQ sample set. Leaves were collected and processed as per the SEQ samples.

2.3. Lipid extraction and purification

2.3.1. Time-series

Total lipid extraction (TLE) of homogenised leaf samples from the time-series study was undertaken at Newcastle University, UK. The method used dichloromethane (DCM):methanol (MeOH) (3:1, v/v) and a CEM MARS 5 microwave system that heated samples to 70 °C over 5 min, holding them at 70 °C for 5 min and then allowing them to cool for 30 min. Excess solvent was removed from the TLE by evaporating under a stream of N₂ (ultra-high

purity; 99.999%). An internal standard of tetratriacontane was added to all samples, which were then passed through a pre-conditioned silica gel column (35–70 mesh size), with the non-polar fraction eluted using *n*-hexane (grade >99.8%). The non-polar fraction then underwent solid phase extraction using a Bond Elut SCX cartridge to separate saturated and unsaturated compounds.

n-Alkane (*n*-C₁₈ to *n*-C₃₃) concentrations were quantified using an HP 5890 Series 2 Gas Chromatograph-Flame Ionization Detector (GC-FID) with a HP1 column (50 m × 0.32 mm i.d. × 0.17 μm film thickness) with a flow rate of 2 mL/min of helium carrier gas. A 1 μl aliquot of sample was injected using a temperature programme that increased the temperature from 50 °C to 300 °C at a rate of 6 °C/min. Compounds were identified using a Thermo Finnigan Trace gas chromatograph–mass spectrometer (GC–MS) with a HP1 column (50 m × 0.32 mm i.d. × 0.17 μm film thickness) with a flow rate of 2 mL/min in a helium carrier gas. A 1 μl aliquot of sample was injected into a programmable temperature vaporising injector system at 270 °C in split-less mode with GC temperature ramped up from 50 °C to 300 °C at a rate of 6 °C/min. Concentrations were normalised to total dry weight of leaf material extracted and are reported as μg/g dry leaf material.

2.3.2. Spatial studies: SEQ and QLD

Preparation of samples from SEQ and QLD took place at the University of Adelaide, Australia. Ground plant material from each sample

was subjected to solvent extraction by being sonicated for 15 min in 7 mL of DCM:MeOH (9:1, v/v). The mixture was then decanted through ashed glass fibre filters. Sonication and filtration were conducted in triplicate on the same sample material, with extracts combined. Excess solvent was removed from the TLE by evaporating under a stream of N₂ (ultra-high purity; 99.999%). The total lipid extract (TLE) was fractionated using short column chromatography with ~0.5 g of activated silica gel (35–70 mesh size). The non-polar fraction, that included *n*-alkanes, was eluted with 4 mL of *n*-hexane (Optima™ grade, Fisher Scientific), followed by elution of the polar fraction with 4 mL DCM:MeOH (1:1, v/v). The non-polar fraction was then evaporated under N₂ and redissolved in 100 µL of *n*-hexane spiked with 10 µg/mL of 1,1'-binaphthyl as internal standard. A quantitation standard was prepared by dilution of a Certified Reference Material (C₇-C₄₀ Saturated Alkanes Standard, Supelco 49452-U) to a concentration of 10 µg/mL and spiked with 10 µg/mL of 1,1'-binaphthyl internal standard for concurrent analysis with the sample batch.

n-Alkane characterisation was undertaken using a PerkinElmer Clarus 500 GC-MS, with an SGE CPSil-5MS (30 m × 0.25 mm ID × 0.25 µm film thickness) capillary column with helium carrier gas with a flow rate of 1 mL/min. A 1 µL aliquot of sample was injected at a temperature of 300 °C. The oven temperature program was 50 °C, held for one min, prior to an 8 °C/min ramp to 340 °C and a final hold of 7.75 min. The mass spectrometer was scanned from 45 to 500 Da. Concentrations of *n*-alkane homologs from

$n\text{-C}_{25}$ to $n\text{-C}_{33}$ were quantified using PerkinElmer TurboMass analytical software based on response factors of individual n -alkane homologs in the standard against the internal standard. The method was validated against a six-point linearity curve in triplicate with reproducibility assessed by 10 times repeat injection ($R^2 > 0.98$, $\pm 3\%$ reproducibility). Concentrations were normalised as per the time-series data.

2.4. Time-averaged climate data

Climate data utilised in this study were derived from the SILO (Scientific Information for Land Owners) database (Jeffrey et al., 2001). Daily climate variables (total precipitation and maximum temperature) were interpolated for sample locations from daily climate observations at proximal observation sites using the DataDrill system (Jeffrey et al., 2001). Growing season climate (June–November, inclusive) was calculated for both the temporal and spatial studies with a mean calculated for three full growing seasons prior to sample collection. Climate averages for three growing seasons were utilised for this study due to the leaf life span of the species – i.e. 2–4 years (Van et al., 2002) – and follows the approach of Tibby et al. (2016). Growing season precipitation at Carbrook Wetlands averaged for the three years prior to sample collection ranged from 280 mm to 543 mm, while maximum daily temperature averaged across the three previous growing seasons ranged from 22.8 °C to 23.6 °C. At SEQ sites, growing season precipitation ranged from 269 mm to 558 mm, with growing

season temperature ranging from 22.3 °C to 24.5 °C. Growing season precipitation for QLD sites, where Cape York sites were included, ranged from 89 mm to 558 mm, with growing season temperature ranging from 22.3 °C to 33.2 °C (Fig. 2).

2.5. Leaf wax characteristic calculations and statistical analysis

Concentration, average chain length (ACL), Norm31 and dispersion were calculated for *n*-alkane chain lengths *n*-C₂₅ to *n*-C₃₃, inclusive. With the exception of concentration, all variables are dimensionless metrics of *n*-alkane distribution variability. ACL is the weighted mean of odd long-chain *n*-alkane concentrations and quantifies the dominant long chain *n*-alkane homolog in a sample (Bush and McInerney, 2013, 2015). Norm31 is the normalized ratio of the *n*-C₃₁ *n*-alkane homolog to the *n*-C₂₉ *n*-alkane homolog, and is a parameter that is environmentally sensitive (Carr et al., 2014). Dispersion is a measure of how narrowly distributed *n*-alkane chain lengths are around a mean (Dodd and Afzal-Rafii, 2000). Formulae for these metrics are as follows:

$$ACL = \frac{\sum(n \times C_n)}{\sum(C_n)} \quad 1.$$

where *n* is the odd carbon chain length and *C_n* is the concentration of the *n*-alkane with *n* carbon atoms (Bush and McInerney, 2015).

$$Norm31 = \frac{n-C_{31}}{n-C_{29} + n-C_{31}} \quad 2.$$

where $n\text{-C}_{31}$ and $n\text{-C}_{29}$ are the concentrations of those n -alkane homologs (Carr et al., 2014).

$$\text{Dispersion} = \sum [p_n(n - \text{ACL})^2] \quad 3.$$

where n is n -alkane chain length and p_n is the proportional concentration of that chain length relative to the total concentration of n -alkanes (Dodd and Afzal-Rafii, 2000).

We utilised redundancy analysis (RDA) in the 'Vegan' package (Oksanen et al., 2017) in R (R Core Team, 2016) to determine the extent to which our environmental variables (rainfall and temperature) explain patterns in the proportional concentration of n -alkanes ($\text{C}_{25}\text{--}\text{C}_{33}$) in the spatial studies. Redundancy analysis is a multivariate technique for examining species–environment relationships. It is equivalent to Principal Components Analysis with the important distinction being that the axes represent combinations of environmental variables (Lepš and Šmilauer, 2003). Redundancy analysis is an appropriate technique to use where the response of the objects (in this case the n -alkane characteristics) are linear in relation to the environmental variables. To determine whether a linear method was appropriate to use we undertook a detrended correspondence analysis in the 'Vegan' package in R (Oksanen et al., 2017), which showed the gradient length of the data set was less than two, indicating linear-based RDA was an appropriate methodology (ter Braak and Prentice, 2004).

Statistical tests were utilised to determine the equality of variance between n -alkane characteristics from the SEQ and time-series studies, to

compare variance through time at a single site and across space at a single time. The Shapiro-Wilk test in base R (R Core Team, 2016) was used to check for normality in the distributions of ACL, concentration, Norm31 and dispersion from each of the SEQ and time-series studies. In most cases, distributions were not normal; because of this it was decided to use Levene's test to determine homogeneity of variance. This was undertaken using the 'leveneTest' function in the 'Car' package (Fox and Weisberg, 2011) in R (R Core Team, 2016). Lastly, we undertook a series of linear regressions between climate and leaf wax *n*-alkane characteristics using $p < 0.05$ as our measure of statistical significance.

3. Results

3.1. Time-series

For samples from the time-series study (Table 1), total concentration of *n*-alkanes (*n*-C₂₅ to *n*-C₃₃ summed) ranged from 47.1 to 1170 µg/g dry leaf (mean: 182; σ : 169). ACL minima and maxima were 28.9 and 30.2 (mean: 29.7; σ : 0.27). Norm31 ranged from 0.50 to 0.77 (mean: 0.67; σ : 0.06).

Dispersion varied between 2.4 and 5.6 (mean: 3.9; σ : 0.57). Total concentration, ACL and Norm 31 did not show statistically significant linear correlations with either of growing season precipitation or temperature (Fig. 3). Dispersion had a significant, though weak, linear correlation with growing season precipitation ($R = 0.33$, $p = 0.028$) and no correlation with growing season temperature (Fig. 3). Note that absolute values of results

from this aspect of the study cannot be directly compared with the SEQ and QLD datasets below, as a result of different sampling strategies and *n*-alkane extraction methods used.

3.2. South-east Queensland (SEQ)

In samples from SEQ (Table 2), the total concentration of *n*-alkanes (*n*-C₂₅ to *n*-C₃₃ summed) ranged from 2.9 to 287 µg/g dry leaf (mean: 34.5; σ : 59.7). ACL ranged from 29.0 to 30.3 (mean: 29.7; σ : 0.32), and Norm31 from 0.52 to 0.81 (mean: 0.66; σ : 0.07). Values of dispersion varied from 1.0 to 6.2 (mean: 4.2; σ : 1.15). For SEQ sites, only Norm31 had statistically significant, but weak, linear correlations with both growing season precipitation and growing season temperature ($R = -0.45$, $p = 0.043$ and $R = 0.45$, $p = 0.041$, respectively) (Fig. 4). Redundancy analysis (Supplementary Fig. S1) indicated that variance in *n*-alkane characteristics of samples from SEQ sites was primarily explained by axis one (constrained to be a combination of the influence of growing season precipitation and temperature, and hence aridity), but the variance explained was low (< 10%). Axis 2 summarises the dominant hypothetical gradient unrelated to the measured variables and explained less than 1% of the variance.

3.3. Cross-Queensland (QLD)

For samples from the QLD sites (Table 2), the summed concentration of the suite of *n*-alkane homologs from *n*-C₂₅ to *n*-C₃₃ ranged from 2.9 to 411

$\mu\text{g/g}$ dry leaf (mean: 66.5; σ : 102). ACL ranged from 29 to 31.5 (mean: 29.9; σ : 0.65). Norm31 ranged from 0.52 to 0.94 (mean: 0.7; σ : 0.12). Values of dispersion varied from 1 to 6.2 (mean: 3.7; σ : 1.43). Redundancy analysis on spatial data across QLD sites (Supplementary Fig. S2) showed that 44% of variance in the *n*-alkane characteristics was explained by axis one, with this axis representing aridity. Axis 2, which summarises the dominant hypothetical gradient unrelated to the measured variables, explained less than 1% of the variance. Concentration, ACL, Norm31 and dispersion all displayed strong correlations with growing season precipitation ($R = -0.61$, $p = 0.001$; $R = -0.78$, $p < 0.001$; $R = -0.8$, $p < 0.001$; $R = 0.64$, $p < 0.001$; respectively), and growing season temperature ($R = 0.62$, $p < 0.001$; $R = 0.86$, $p < 0.001$; $R = 0.83$, $p < 0.001$; $R = -0.68$, $p < 0.001$; respectively) (Fig. 5). When the Cape York samples (samples CY1, 2, 3, 4) in this aspect of the study are considered alone, no significant linear correlations between *n*-alkane characteristics and either of growing season precipitation or temperature are observed (Fig. 5).

3.4. Levene's test of homogeneity of variance (SEQ and time-series)

Comparison of variance between the SEQ and time-series studies using Levene's test resulted in p -values for the *n*-alkane characteristics ACL, concentration and Norm31 of 0.71, 0.21 and 0.64, respectively. Hence homogeneity of variance of these *n*-alkane characteristics exists between the SEQ and time-series studies. Comparison of *n*-alkane dispersion variance

between the SEQ and time-series studies using Levene's test resulted in a p value of 0.01, indicating significant heterogeneity of variance.

4. Discussion

We measured n -alkane characteristics of modern *M. quinquenervia* leaves in a 11 year time-series, as well as across small- and large-scale climate transects, to explore the potential for calibration of these characteristics for novel single species palaeoclimatic reconstructions in south-east Queensland (Tibby et al., 2016; Barr et al., 2019). The single species approach undertaken in this study controlled for intrinsic differences in n -alkane production within plant communities that have the potential to significantly bias modern n -alkane characteristic-climate calibrations (Vogts et al., 2009; Diefendorf et al., 2011, 2015; Carr et al., 2014; Freeman and Pancost, 2014; Garcin et al., 2014; Diefendorf and Freimuth, 2017; Jansen and Wiesenberg, 2017; Howard et al., 2018). This was crucial for assessment of n -alkane characteristic responsiveness to climate, and to constrain whether leaf wax n -alkane characteristics in *M. quinquenervia* reflect plastic responses to short-term climate changes or whether they reflect fixed traits associated with different ambient climate conditions (Bender et al., 2017).

Most n -alkane characteristics of *M. quinquenervia* (concentration, ACL, Norm31) did not respond plastically to climate variability in the 11-year time-series from Carbrook Wetlands (Fig. 3). In the smaller regional

sample set of south-east Queensland (SEQ) concentration, ACL, and dispersion are also not significantly correlated with climate (Fig. 4).

Similarly, *n*-alkane characteristics of samples from the Cape York region, when considered alone as a regional sample set, do not correlate with climate, although the sample size is small (Fig. 5).

Concentration, ACL and Norm31 displayed homogenous variance between the time-series and SEQ datasets, suggesting that variation in these characteristics at the site level is as large as at the regional level. While most of the variation in the temporal and SEQ data is not explained by climate, weak correlations were observed between dispersion and precipitation in the time-series as well as between Norm31 and both climate variables in SEQ. Therefore, overall *n*-alkane characteristics are unresponsive or only weakly responsive to climate variability in a plastic sense when examined in a time-series at one location, and across relatively small distances (~150 km). This lack of responsiveness of *n*-alkane characteristics to climate in the time-series and SEQ could be influenced by hydrological buffering in the wetland habitat of this species (Ireland et al., 2002). However, carbon isotope ratios of leaves from this same time-series show strong correlations with rainfall through the impact of water stress on carbon isotope fractionation (Tibby et al., 2016). The carbon isotope data demonstrates that these trees are sensitive to climatic fluctuations through time. Therefore, the lack of response in *n*-alkane characteristics observed here indicates a lack of plasticity.

In contrast, the QLD data show significant linear relationships between climate and all *n*-alkane characteristics measured. The linear relationships observed are largely a function of the addition of Cape York samples from sites with markedly lower growing season precipitation and higher growing season temperature than sites in SEQ. These samples display distinctly higher concentration, ACL, Norm31 and lower dispersion than samples from SEQ, and this is suggestive of two broad distinct groups of *M. quinquenervia* in terms of their *n*-alkane production. The assertion of geographic groupings of *n*-alkane characteristics in *M. quinquenervia* is further supported by redundancy analysis of samples from SEQ and QLD (Supplementary Figs. S1 and S2). When Cape York samples are included in the redundancy analysis, 44% of variance in *n*-alkane characteristics can be explained by aridity, compared to less than 10% for only SEQ samples.

The observations in the QLD aspect of the study are consistent with the functional role of leaf wax *n*-alkanes proposed by the barrier membrane model (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994; Riederer and Schreiber, 1995; Jetter and Riederer, 2016). More abundant, longer and more narrowly distributed *n*-alkanes are observed at sites with lower growing season rainfall and higher temperatures, where mitigating non-stomatal water loss would be critical. Similar positive correlations have been observed between ACL and growing season temperature in a study that examined within-species variation in *Acer rubrum* and *Juniperus*

virginiana from a spatial transect in North America (Tipple and Pagani, 2013).

Taken together, the data suggest that leaf wax *n*-alkane characteristics of *M. quinquenervia* do not respond plastically to climate, but instead appear fixed. Yet, *n*-alkane characteristics differ between south-east Queensland and Cape York in ways that are consistent with the barrier-membrane model. These fixed differences could reflect natural selection for less permeable cuticles in more arid regions. A number of studies have interpreted differences in *n*-alkane production in plants as a result of adaptation to different climates of populations of a single species, rather than a plastic response (Dodd et al., 1998; Dodd and Afzal-Rafii, 2000; Dodd and Poveda, 2003; Rajčević et al., 2014). Greenhouse and common garden experiments indicate that leaf wax compound profiles reflect genetic determination more strongly than short-term environmental influence (Gosney et al., 2016; Bender et al., 2017). To demonstrate that leaf wax *n*-alkane characteristics in *M. quinquenervia* are fixed through genetic control requires further research (e.g., genetic sequencing, common garden experiments). However, the evidence is suggestive that genetics could be the main control on *n*-alkane production in leaves of *M. quinquenervia*.

The results of this study have implications for approaches using *n*-alkane abundance and distributions for palaeoclimatic reconstructions. Any changes in *n*-alkane characteristics of sub-fossil leaves of *M. quinquenervia* would likely not reflect plastic responses to small-scale variations in

climate, but instead could represent much larger and longer sustained climatological shifts. This distinction has important ramifications for the interpretation of *n*-alkane characteristics as a proxy for variation in climate through time, particularly in the context of the scale of climatological shifts able to be perceived from geological records.

5. Conclusions

Leaf wax *n*-alkane characteristics have been examined in leaves of *Melaleuca quinquenervia* on both spatial and temporal climate gradients. We observed weak or no correlation between both growing season precipitation and temperature and *n*-alkane characteristics in proximal samples from south-east Queensland and a time-series of one population, even though climate and *n*-alkane characteristics vary substantially in both cases. We observed longer and more narrowly distributed leaf wax *n*-alkanes in samples of *M. quinquenervia* from a markedly warmer and dryer growing season climate regime, which is consistent with the prevailing model for the function of leaf-wax *n*-alkanes in preventing water loss. We interpret our results as reflecting fixed responses to broad climate regimes as opposed to plastic responses to regional microclimate spatially and through time. These results have implications for interpreting *n*-alkane characteristics of sub-fossil leaves, with changes in these in sedimentary archives likely reflecting large climatological shifts, rather than short-term climate variability.

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Figure captions

Fig. 1. A modified Köppen climate classification map of Australia (Peel et al., 2007) with extents of the (a) Cape York and (b) south-east Queensland sampling regions indicated. Time-series, SEQ and QLD components of the study are marked.

Fig. 2. Violin plots showing the range of (a) growing season precipitation and (b) growing season temperature for each of the time-series, SEQ and QLD aspects of the study. Black symbols represent the mean for each range.

Fig. 3. *n*-Alkane characteristics and growing season precipitation (a, b, c, d) and growing season temperature (e, f, g, h) through time from Carbrook Wetlands. Results of linear regression modelling between each *n*-alkane characteristic and precipitation or temperature is indicated at the top right of each panel. The thin black line represents the *n*-alkane characteristic data series, while the wide gray line represents climate variables.

Fig. 4. *n*-Alkane characteristics versus growing season precipitation (a, b, c, d) and growing season temperature (e, f, g, h) from our south-east Queensland study (SEQ), with results of linear regression modelling indicated.

Fig. 5. *n*-Alkane characteristics versus growing season precipitation (a, b, c, d) and growing season temperature (e, f, g, h) from the cross-Queensland study (QLD). Open symbols indicate samples from SEQ study, with closed symbols representing Cape York samples augmenting the SEQ data. Summary statistics from linear regressions (correlation coefficient and *p*-value) are indicated for all samples (black) and only Cape York samples (gray, italics).

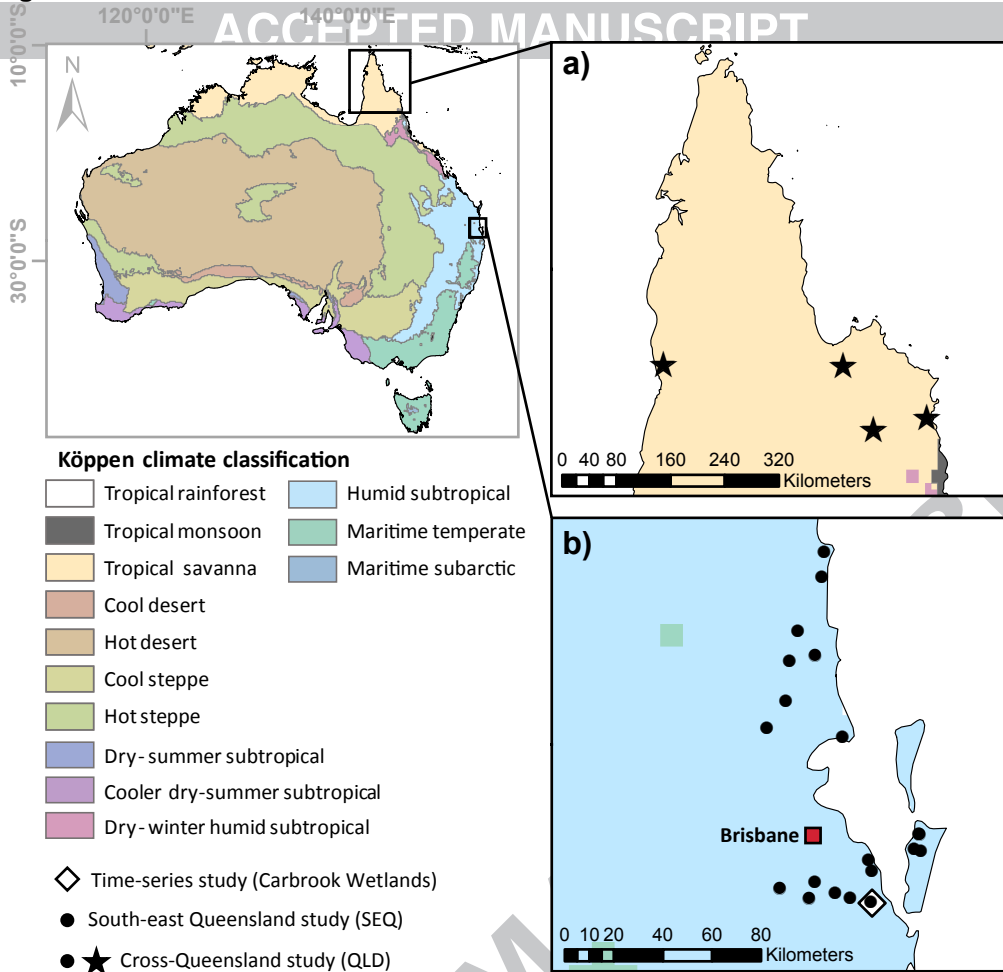
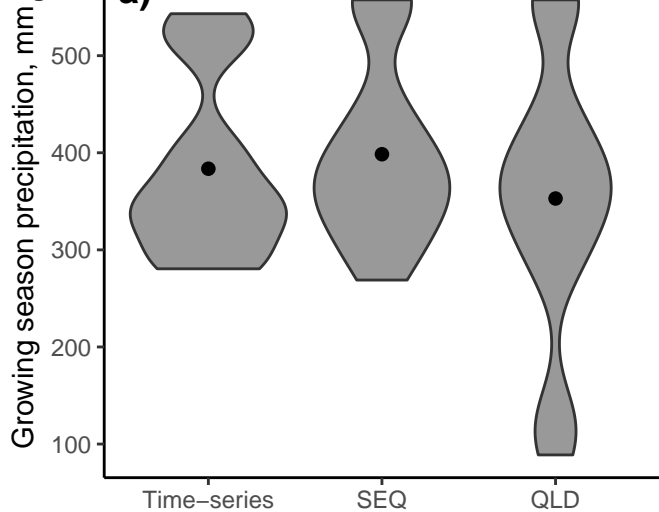
Figure 1

Figure 2 a)



b)

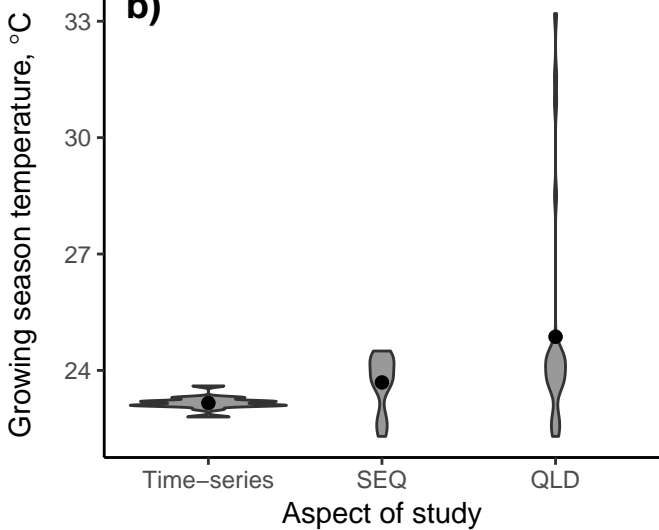


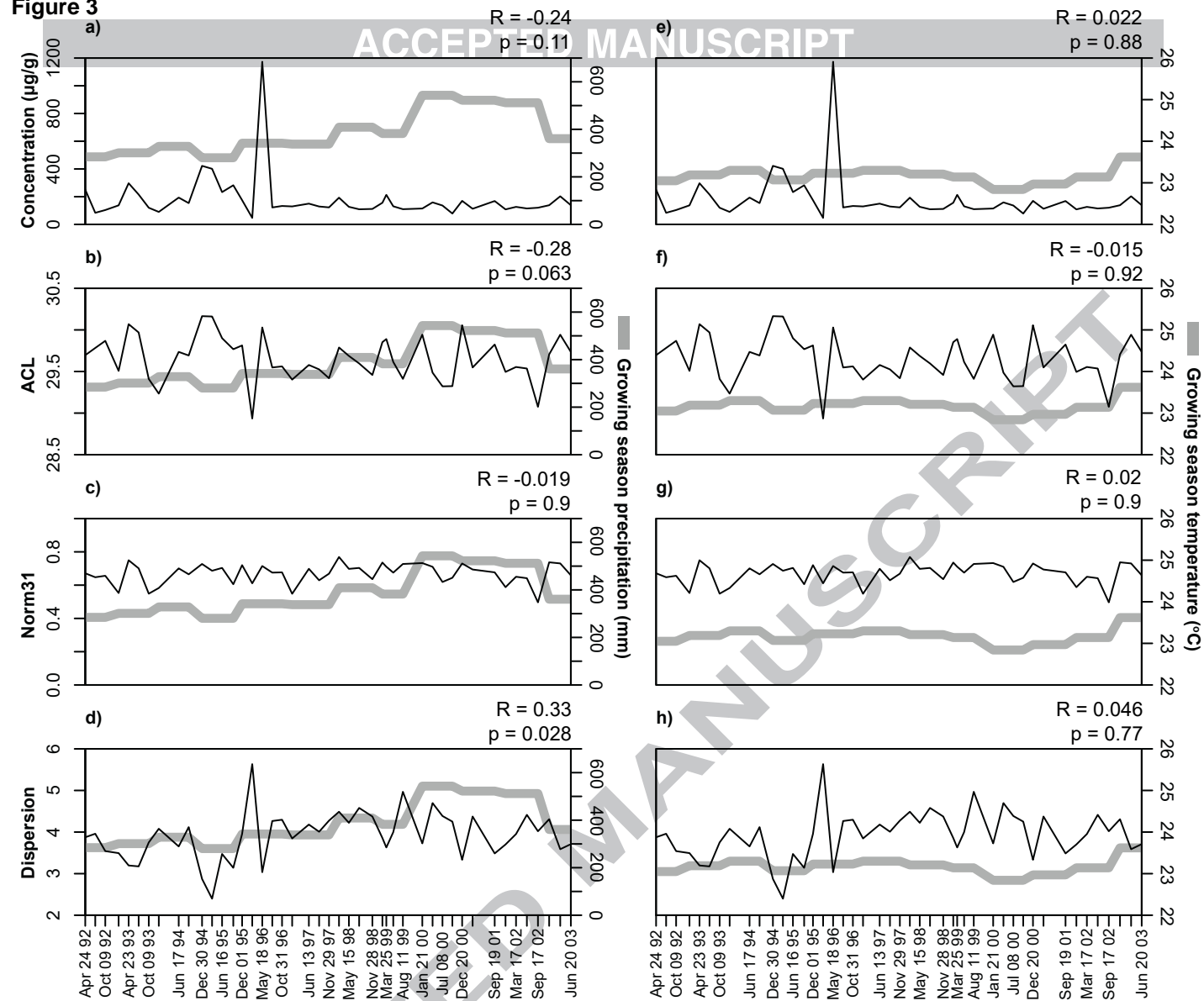
Figure 3

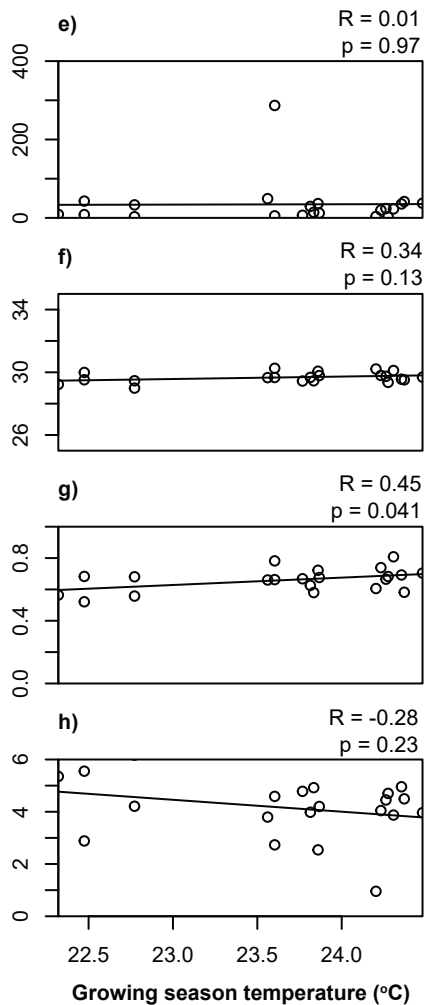
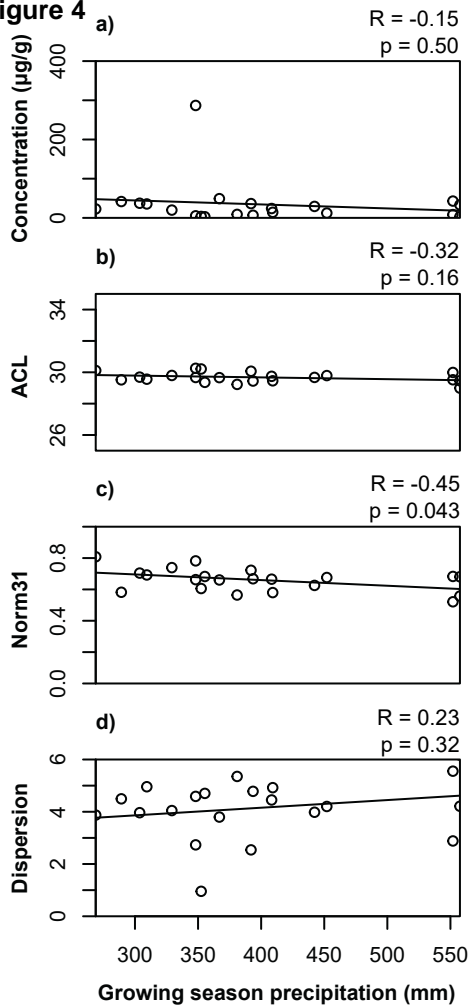
Figure 4

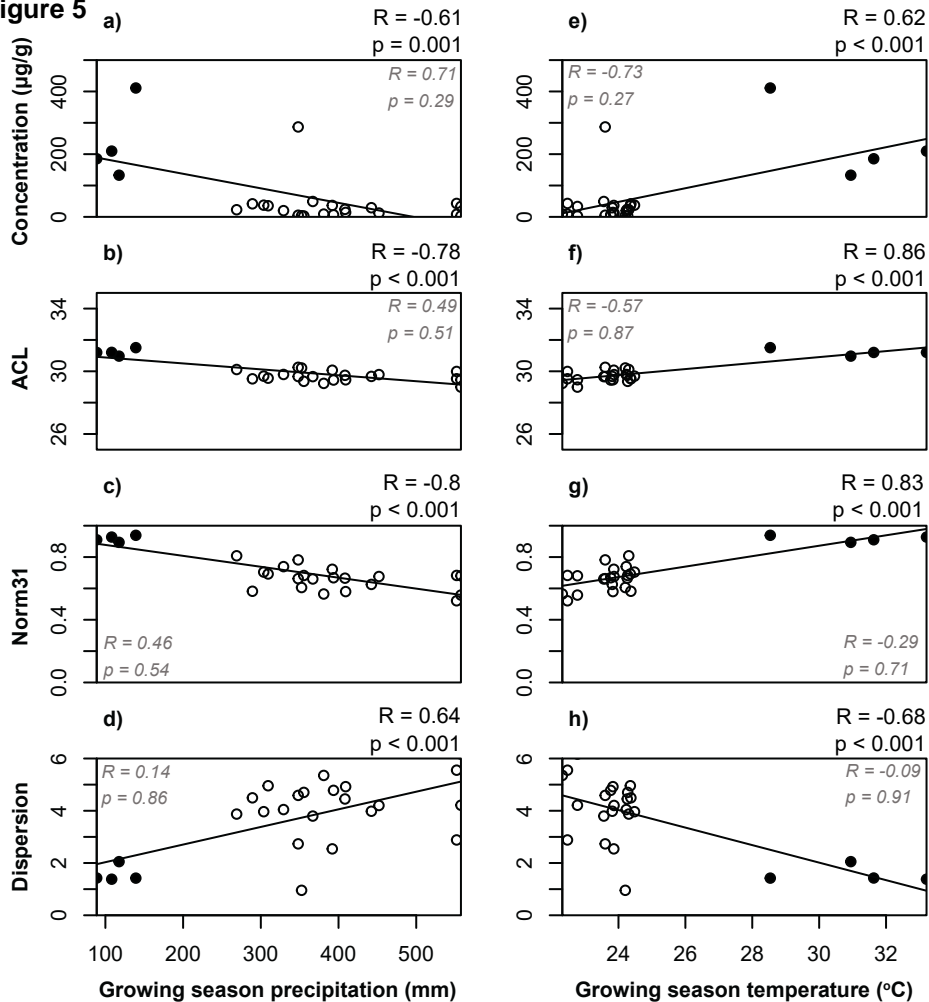
Figure 5

Table 1. Dates of sample collection, climate variables and leaf wax *n*-alkane characteristics for samples in the time-series study at Carbrook Wetlands.

Date of sample collection	Mean growing season total precipitation (mm)	Mean daily growing season temperature (°C)	ACL (C ₂₅ –C ₃₃)	Concentration (µg/g dry leaf, C ₂₅ –C ₃₃)	Norm31	Dispersion (C ₂₅ –C ₃₃)
24.4.92	284.4	23.1	29.7	250.6	0.7	3.9
17.7.92	284.4	23.1	29.8	83.6	0.6	4.0
9.10.92	284.4	23.1	29.9	103.7	0.7	3.5
29.1.93	301.2	23.2	29.5	137.5	0.6	3.5
23.4.93	301.2	23.2	30.1	297.0	0.7	3.2
16.7.93	301.2	23.2	30.0	215.6	0.7	3.2
9.10.93	301.2	23.2	29.4	120.1	0.5	3.8
1.1.94	328.3	23.3	29.2	90.1	0.6	4.1
17.6.94	328.3	23.3	29.7	193.5	0.7	3.7
9.9.94	328.3	23.3	29.7	153.6	0.7	4.1
30.12.94	280.5	23.1	30.2	422.3	0.7	2.9
24.3.95	280.5	23.1	30.2	401.1	0.7	2.4
16.6.95	280.5	23.1	29.9	233.0	0.7	3.5
18.9.95	280.5	23.1	29.8	282.3	0.6	3.1
1.12.95	341.7	23.2	29.8	174.7	0.7	4.0
24.2.96	341.7	23.2	28.9	47.1	0.6	5.6
18.5.96	341.7	23.2	30.0	1173.1	0.7	3.0
10.8.96	341.7	23.2	29.5	122.2	0.7	4.3
31.10.96	341.7	23.2	29.6	132.9	0.7	4.3
24.1.97	337.9	23.3	29.4	129.6	0.5	3.8
13.6.97	337.9	23.3	29.6	149.8	0.7	4.2
7.9.97	337.9	23.3	29.5	129.1	0.6	4.0
29.11.97	337.9	23.3	29.4	122.7	0.7	4.3

21.2.98	409.1	23.2	29.8	193.0	0.8	4.5
15.5.98	409.1	23.2	29.7	126.8	0.7	4.2
9.8.98	409.1	23.2	29.6	109.1	0.7	4.6
28.11.98	409.1	23.2	29.5	111.6	0.6	4.4
20.2.99	382.4	23.1	29.9	156.2	0.7	3.9
25.3.99	382.4	23.1	29.9	213.3	0.7	3.6
22.5.99	382.4	23.1	29.6	130.6	0.7	4.0
11.8.99	382.4	23.1	29.4	109.8	0.7	5.0
21.1.00	543.2	22.8	29.9	114.6	0.7	3.7
16.4.00	543.2	22.8	29.5	158.9	0.7	4.7
8.7.00	543.2	22.8	29.3	136.1	0.6	4.4
29.9.00	543.2	22.8	29.3	78.2	0.6	4.3
20.12.00	522.4	23.0	30.1	169.7	0.7	3.3
18.3.01	522.4	23.0	29.5	112.9	0.7	4.4
19.9.01	522.4	23.0	29.8	168.7	0.7	3.5
19.12.01	512.0	23.1	29.5	108.3	0.6	3.7
17.3.02	512.0	23.1	29.6	126.5	0.7	4.0
16.6.02	512.0	23.1	29.5	115.0	0.6	4.4
17.9.02	512.0	23.1	29.1	120.3	0.5	4.0
20.12.02	360.8	23.6	29.7	138.4	0.7	4.3
23.3.03	360.8	23.6	29.9	202.7	0.7	3.6
20.6.03	360.8	23.6	29.7	138.6	0.7	3.7

Table 2. Geographic locations, climate variables and leaf wax *n*-alkane characteristics for samples in the SEQ and QLD studies.

Sample name	Longitude E (°)	Latitude S (°)	Mean growing season total precipitation (mm)	Mean daily growing season temperature (°C)	ACL (C ₂₅ –C ₃₃)	Concentration (µg/g dry leaf, C ₂₅ –C ₃₃)	Norm31	Dispersion (C ₂₅ –C ₃₃)
G1a	153.1	27.7	348.1	23.6	30.3	286.8	0.8	2.7
G2a	153.2	27.7	355.4	24.3	29.4	2.9	0.7	4.7
G3a	153.3	27.7	352.5	24.2	30.2	3.6	0.6	1.0
G4a	153.3	27.6	392.0	23.9	30.1	36.4	0.7	2.5
G5a	153.3	27.5	366.9	23.6	29.7	49.1	0.7	3.8
G6a	153.1	26.8	452.0	23.9	29.8	12.2	0.7	4.2
G7a	153.0	26.8	393.6	23.8	29.4	6.7	0.7	4.8
G8a	153.0	26.9	381.0	22.3	29.2	8.9	0.6	5.4
G9a	152.9	27.0	289.2	24.4	29.5	41.6	0.6	4.5
G10a	153.2	27.1	348.1	23.6	29.7	5.2	0.7	4.6
J1a	153.1	27.6	268.9	24.3	30.1	22.8	0.8	3.9
J2a	152.9	27.6	303.7	24.5	29.7	37.3	0.7	4.0
J3a	153.0	27.7	309.4	24.4	29.6	35.5	0.7	5.0
J4a	153.0	26.7	408.3	24.3	29.7	23.9	0.7	4.5
J5a	153.0	26.3	329.2	24.2	29.8	19.9	0.7	4.0
J6a	153.1	26.4	409.2	23.8	29.5	14.2	0.6	4.9
J7a	153.1	26.5	442.3	23.8	29.7	29.2	0.6	4.0
WL1a	153.4	27.4	557.5	22.8	29.0	2.9	0.6	6.2
WL2a	153.4	27.4	557.5	22.8	29.5	33.2	0.7	4.2
SL1a	153.5	27.5	552.0	22.5	30.0	42.9	0.7	2.9
BL1a	153.4	27.5	552.0	22.5	29.5	8.3	0.5	5.6
CY1a	141.7	14.8	88.8	31.6	31.2	185.4	0.9	1.4
CY2a	144.5	15.6	139.2	28.5	31.5	411.0	0.9	1.4
CY3a	144.1	14.8	117.6	30.9	31.0	132.9	0.9	2.1
CY4a	145.2	15.5	108.1	33.2	31.2	209.8	0.9	1.4

Highlights

n-Alkanes were characterised across both temporal and spatial variations in climate.

Characteristics show little correlation with climate through time at a site.

Characteristics correlate significantly with climate across a large spatial transect.

Correlations are consistent with the functional role of *n*-alkanes.

Sub-fossil leaf wax records are likely to reflect large climatological shifts.