



Heat stress reduces stomatal numbers in *Ginkgo biloba*: Implications for the stomatal method of palaeo-atmospheric [CO₂] reconstruction during episodes of global warming

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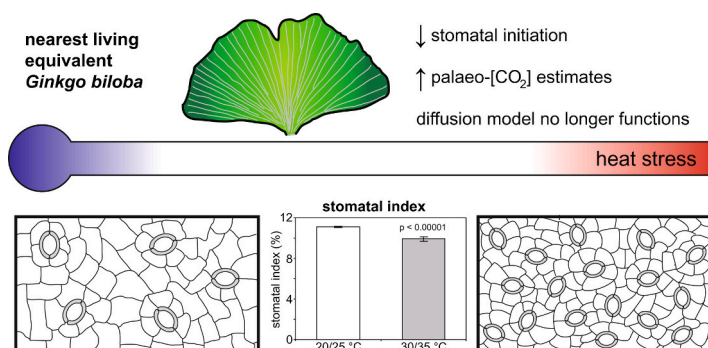
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HIGHLIGHTS

- The 'stomatal method' is widely used to reconstruct palaeo-[CO₂] over Earth history.
- Reliable palaeo-[CO₂] is important during periods of rising global temperatures.
- *Ginkgo biloba* is often used as a nearest living equivalent for fossil Ginkgoales.
- Heat stress induced a reduction in stomatal initiation in *Ginkgo biloba*.
- Measured and modelled stomatal conductance did not correlate under heat stress.
- Stomatal palaeo-[CO₂] reconstructions may be affected by heat stress.

GRAPHICAL ABSTRACT



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ABSTRACT

The stomata of fossil plants are commonly used as proxies to reconstruct palaeo-atmospheric carbon dioxide concentrations (palaeo-[CO₂]). Stomatal reconstruction of palaeo-[CO₂] during global greenhouse periods or episodes of global warming, are particularly important to our understanding of the role of CO₂ as a climate system driver. However, the efficacy of the 'stomatal method' for palaeo-[CO₂] reconstruction depends upon the strength of the inverse relationship between stomatal number and the [CO₂] in which the leaf developed. However, the impact of heat stress on stomatal initiation and development are largely unknown. *Ginkgo biloba*, a living fossil species, seedlings were grown in controlled environment chambers under 20/25 °C and 30/35 °C night/day temperature regimes. Heat stress in the 30/35 °C treatment impaired photosynthetic function, decreased stomatal conductance (G_s), and reduced stomatal index (SI), indicative of lower stomatal initiation. Modelled theoretical G_s did not correlate with observed measured G_s , undermining the utility of palaeo-[CO₂] reconstructions based on stomatal diffusion modelling. The lower stomatal initiation of *G. biloba* leaves from the higher temperature resulted in greater estimates of [CO₂] based on SI values using the nearest living equivalent and SI-[CO₂] transfer function approaches. Heat stress may diminish the effectiveness of the stomatal method in reconstructing palaeo-[CO₂] during intervals of global warming marked by floral turnover in Earth history.

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1. Introduction

The reconstruction of palaeo-atmospheric carbon dioxide concentrations (palaeo-[CO₂]) from the stomata of fossil plants is a widely applied palaeo-climatological proxy (eg. Beerling and Rundgren, 2000; Haworth et al., 2005; Kürschner et al., 2008; Passalia, 2009; Bonis et al., 2010; Jing and Bainian, 2018; Steinthorsdottir et al., 2021). The ‘stomatal method’ is based upon the inverse relationship between the number of stomata on a leaf surface and the atmospheric concentration of carbon dioxide ([CO₂]) in which the leaf developed (Raven and Sprent, 1989; Chaloner and Creber, 1990; Beerling and Chaloner, 1994; Beerling and Royer, 2002). The extended evolutionary history, geographical distribution, and high preservation potential of Ginkgoales has contributed to a large number of palaeo-[CO₂] reconstructions based upon the stomatal frequencies of their fossils (eg. McElwain et al., 1999; Retallack, 2001; Royer et al., 2001; Beerling et al., 2002; Greenwood, 2005; Bainian et al., 2007; Sun et al., 2007; Quan et al., 2009; Smith et al., 2010; Steinthorsdottir et al., 2011; Pieńkowski et al., 2020). The use of the stomatal method to reconstruct palaeo-[CO₂] from the stomatal characters of fossil Ginkgoales relies upon the strength and consistency of the relationship between atmospheric [CO₂] and the frequency of stomata on the leaf surface. A recent examination of historical herbarium specimens of the only extant member of the Ginkgoaceae, *Ginkgo biloba* L. (a species considered as a living fossil), indicates that the relationship between stomatal number and atmospheric [CO₂] holds in different climatic regions (Steinthorsdottir et al., 2022). However, it remains unclear whether stomatal initiation and leaf expansion (key components of stomatal index and density - parameters that are widely used in stomatal reconstructions of palaeo-[CO₂]) are affected by heat stress, and the potential impact of any influence of heat stress on the reliability of the stomatal method.

Stomata are the tiny pores covering a leaf surface through which gas exchange occurs. The selective pressure exerted by the need to optimise water use efficiency by balancing photosynthetic CO₂-uptake against transpirative water loss led to physiological and morphological responses to optimise stomatal control (Robinson, 1994; Franks and Beerling, 2009; Haworth et al., 2021). In plants termed ‘stomatal responders’, this has led to the occurrence of an inverse relationship between stomatal number and [CO₂]: in effect, under conditions of high [CO₂], similar rates of CO₂-uptake can be achieved with fewer stomata, and a correspondingly lower rate of water loss, by reducing stomatal initiation in developing leaves (Körner, 1988; Caine et al., 2019). Mature leaves signal the internal sub-stomatal [CO₂] (C_i) to developing leaves (Lake et al., 2001) where stomatal initiation and epidermal patterning is regulated by genes such as ERECTA, EPIDERMAL PATTERNING FACTORS, TOO MANY MOUTHS and STOMAGEN (Masle et al., 2005; Hara et al., 2007; Hunt and Gray, 2009; Tripathi et al., 2016). This relationship between stomatal number and [CO₂] has been used to infer the [CO₂] in which a leaf developed, enabling fossil plants to serve as proxies of palaeo-[CO₂] (eg. Rundgren and Beerling, 1999; Royer et al., 2001; Smith et al., 2010). It is worth noting that not all plant species adjust stomatal number in response to [CO₂] (Haworth et al., 2011; Haworth et al., 2016) or in the same direction (Hu et al., 2015). Stomatal number is primarily gauged as the parameter stomatal density (SD), where the number of stomata per unit area of epidermis is quantified. However, SD is susceptible to the effect of cellular expansion, meaning that factors reducing cell expansion, such as water deficit, can in turn influence SD in addition to [CO₂] (Beerling, 1999). The stomatal index (SI) nullifies the impact of leaf expansion by quantifying the number of stomata as a percentage of surrounding epidermal cells to indicate the rate of stomatal initiation, and thus produce a clearer signal of [CO₂] during leaf development (Beerling, 1999; Beerling and Royer, 2002). Stomatal index can be used to gauge the [CO₂] in which a leaf developed through the use of a calibration to the same or a nearest living equivalent species (McElwain and Chaloner, 1996) or the use of a SI – [CO₂] transfer function constructed from the stomatal responses of a

living species to [CO₂] from historical herbarium (eg. Kürschner et al., 2008) and experimental [CO₂] enrichment datasets (eg. Retallack, 2001; Royer et al., 2001). It is worth noting that a number of reconstructions of palaeo-[CO₂] based on the stomata of fossil plants utilise the SD of the fossil plant rather than SI values, presumably due to the preservational state of the fossil plant cuticle precluding clear delineation of the epidermal cells (eg. Steinthorsdottir et al., 2016). The use of diffusion based models to approximate the palaeo-[CO₂] in which a leaf developed require quantification of SD and the dimensions of the stomatal pore to model leaf gas exchange potential (Konrad et al., 2008; Franks et al., 2014). Stomatal size (SS) and density often shift in tandem in response to changes in plant growth conditions (Haworth et al., 2023), with an inverse relationship between SS and SD observed in living and fossil plants (Hetherington and Woodward, 2003; Franks and Beerling, 2009). Sensitivity testing of a diffusion based model of leaf gas exchange suggests that its efficacy would likely be diminished at high temperatures (Konrad et al., 2021). Nevertheless, the effectiveness of all of the stomatal method to reconstruct palaeo-[CO₂] is dependent upon [CO₂] being the primary signal influencing stomatal initiation and morphological development.

The Ginkgoales have a temporally extended and spatially extensive fossil record (Seward, 1919; Tralau, 1968). The sole extant member of the Ginkgoales, *G. biloba*, is considered a ‘stomatal responder’ due to historical herbarium (Chen et al., 2001; Royer et al., 2001; Retallack, 2002) and experimental [CO₂] enrichment studies (Beerling et al., 1998; Royer et al., 2001; Haworth et al., 2013) showing stomatal morphological adjustment to [CO₂]. This has led to the extensive use of fossil Ginkgoales as stomatal palaeo-[CO₂] proxies; particularly at key intervals marked by perturbation to global climate and biogeochemical cycling (McElwain et al., 1999; Beerling et al., 2002; Bonis et al., 2010; Smith et al., 2010; Steinthorsdottir et al., 2011; Wu et al., 2016). Attempts have been made to enhance the strength of the Ginkgoales stomatal palaeo-[CO₂] record by analysis of the stomatal morphological relationship of [CO₂] of *G. biloba* under a range of climatic conditions. The SI values of *G. biloba* were largely unaffected by climate in samples collected from China and England. However, *G. biloba* leaves categorised as sun leaves exhibited 2.2 to 5.5 % higher SI values than their shade counterparts (Sun et al., 2003). Steinthorsdottir et al. (2022) examined the stomatal morphological characteristics of *G. biloba* leaves from 141 trees in 18 countries representing a comprehensive examination of the climatic range where the species can be cultivated. Steinthorsdottir et al. (2022) noted that the three methods to reconstruct [CO₂] from the stomatal parameters of *G. biloba* were not significantly affected by temperature, precipitation or seasonality. These findings would suggest that stomatal initiation in *G. biloba* is largely the result of [CO₂] during leaf development, giving confidence to the use of Ginkgoales based stomatal reconstructions of palaeo-[CO₂] over deep-time. However, these studies consider climatic variations that might be considered to be within the tolerance limits of *G. biloba* (Bidak et al., 2022; Feng et al., 2023). Many episodes of disruption to the global carbon cycle in Earth history are marked by increases in mean global temperature, with significant regional temperature differences. For example, the Triassic – Jurassic boundary (200 million years ago) was marked by a mean global increase in temperature proposed to be in the range of 2.0 to 2.5 °C (Beerling and Berner, 2002), 2.5 to 5.0 °C (Berner and Kothavala, 2001) or 3.0 to 4.0 °C (McElwain et al., 1999) with wider regional variations in temperature (Huynh and Poulsen, 2005). Analysis based on the correlations between adaxial epidermal micro-morphology and the photosynthetic status of *G. biloba* suggests that heat stress may have played a significant role in the loss of physiological function of Late Triassic Ginkgoales from Astartekløft, East Greenland (Haworth et al., 2014). This proposed reduction in photochemical energy usage and increase in non-photochemical quenching corresponded to the regional extinction of Ginkgoales, represented by their absence in the Astartekløft macro-fossil record in the fossil plant bed considered to represent the boundary between the Triassic and Jurassic periods (Harris, 1937; McElwain et al.,

2007). In light of the importance of accurate reconstruction of palaeo-[CO₂] during such warming episodes, the potential influence of heat stress on stomatal initiation, and by extension the clarity of the stomatal signal of [CO₂], should be examined.

Heat stress negatively impacts the carbon and water balance of plants through adverse effects on stomatal control and the biochemical assimilation of CO₂ (Killi et al., 2017). Photosynthesis (P_N) declines at temperatures above optimal levels because, relative to O₂, there is a decrease in the solubility of CO₂ (Jordan and Ogren, 1984), a reduction in the affinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) for CO₂ (Berry and Björkman, 1980), lower RubisCO activity (Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000; Sharkey, 2005), and impaired electron transport through Photosystem II (PSII) (Feller et al., 1998; Killi et al., 2020). Heat stress can induce both increases (Schulze et al., 1973; Urban et al., 2017a) and decreases (Haworth et al., 2018b) in G_s depending upon the pre-existing temperature adaptational status of the plant (Fares et al., 2011; Wang et al., 2016; Killi et al., 2017). A rise in G_s will cause an increase in water loss (Urban et al., 2017b; Marchin et al., 2023), while a reduction in G_s could exacerbate the detrimental effects of heat stress by reducing transpirative cooling (Killi et al., 2017). The reduction in photochemical energy usage (Crafts-Brandner and Salvucci, 2002; Killi et al., 2020), alongside potential reductions in transpirative cooling (Haworth et al., 2018b), can result in photooxidative damage and the upregulation of antioxidant protective mechanisms under heat stress (Killi et al., 2020). The leaves of *G. biloba* exposed to heat stress in a controlled environment chamber study showed reduced leaf area, higher leaf mass per area, and increased leaf construction costs per unit leaf area associated with shifts in foliar composition. Stomatal conductance and non-photochemical quenching declined under heat stress, consistent with a reduction in transpirative cooling and protective energy dissipation as heat (Haworth et al., 2018a). This may suggest that while variations within the climatic tolerance range of *G. biloba* are unlikely to affect stomatal initiation (as observed by Steinhorsdottir et al., 2022), more extreme temperature increases that induce a stress response could prompt a shift in stomatal morphology through stomatal initiation or cellular / leaf expansion effects.

Accurate [CO₂] records during episodes of global warming in the course of Earth history are vital to our understanding of the global climate system and modelling efforts to predict the potential impact of future rises in [CO₂] (Bernier, 1994; van de Wal et al., 2011; Franks et al., 2018). We performed a controlled environment study where *G. biloba* plants were grown under two temperature regimes to experimentally assess any possible effects of heat stress on stomatal characteristics, and the potential impact on palaeo-[CO₂] reconstructions during episodes of pronounced global warming in Earth history (eg. Smith et al., 2010; McInerney and Wing, 2011; Steinhorsdottir et al., 2011; Joachimski et al., 2012). Controlled environment chamber experiments cannot recreate long-term multi-generational heat stress equivalent to that likely to have occurred during intervals of climate perturbation marked by significant global warming such as the Triassic – Jurassic boundary (Pieńkowski et al., 2020; Bos et al., 2023) or Early Eocene Climatic Optimum (Smith et al., 2010). Nevertheless, these experimental studies can enhance our understanding of the impact of specific growth factors on plants that explain patterns observed in the fossil record. To the best of our knowledge, no previous studies have reported on the impact of heat stress on the stomatal morphological responses of *G. biloba*. We hypothesize that heat stress is likely to disrupt stomatal development through impacts on leaf expansion alongside disruption to photosynthetic and photo-protective physiological processes. Any potential disruption to the relationships between stomatal morphology and leaf gas exchange may adversely affect all three methodologies to reconstruct palaeo-[CO₂]. We exposed *G. biloba* to heat stress under controlled environment conditions to: i) assess the potential impact of heat stress on stomatal initiation and morphology; ii) investigate the effect of heat stress on G_s and P_N ; iii) explore possible interactions between modelled

and observed G_s under heat stress and the implications for palaeo-physiological interpretations of fossil plants, and; iv) outline the potential implications of any heat stress effects on stomatal morphology and conductance on the efficacy of stomatal reconstructions of palaeo-[CO₂] during intervals of global warming in Earth history.

2. Materials and methods

2.1. Growth conditions

The impact of temperature on the physiology and stomatal morphology of *G. biloba* was investigated using two large walk-in controlled environment chambers. Details of the controlled environment chambers can be found in Materassi et al. (2005). Both controlled environment chambers operated identical growth conditions with the exception of the temperature regime: a photosynthetic photon flux density (PPFD) of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from metal halide lights for 14 h each day with a one hour ‘ramping’ period at the beginning and end to simulate dawn / dusk, ambient [CO₂] of 406 $\mu\text{mol mol}^{-1}$, and a relative humidity of 60 %. The day / night temperature regimes of the two controlled environment chambers followed changes in PPFD with one chamber operating a 20/25 °C temperature regime (hereafter termed the ‘25 °C treatment’), and the other a 30/35 °C temperature regime (hereafter termed the ‘35 °C treatment’).

Five two-year old *G. biloba* seedlings were placed in each controlled environment chamber. The *G. biloba* seedlings were grown in a 5:1 mixture of compost (COMPO Italia, Cesane Moderno, Italy) and vermiculite in 6 L square pots. *Ginkgo biloba* is deciduous and the seedlings were in their winter dormancy phase when placed in the controlled environment chamber, so that leaf development occurred completely under experimental conditions. To enable free access rates of nutrient uptake, a liquid plant fertilizer (COMPO Concime Universale: N-P-K 7–5–7; B; Cu; Fe; Mn; Mo; Zn) was supplied to the plants weekly. Irrigation of the plants to pot capacity occurred every two-days.

2.2. Leaf physiological analysis

Leaf gas exchange measurements were performed over a two-week period 12 weeks after the *G. biloba* seedlings had been placed into the controlled environment chambers. A PP-Systems Ciras-2 with a PLC6(U) 2 cm² leaf cuvette and LED light unit (90 % red, 10 % blue light) (PP-Systems, Amesbury MA, USA) was used to measure the rates of photosynthesis (P_N), stomatal conductance (G_s), and the sub-stomatal internal [CO₂] (C_i) of individual leaves under cuvette conditions of: 400 $\mu\text{mol mol}^{-1}$ [CO₂], 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 60 % relative humidity and a leaf temperature of either 25 or 35 °C depending upon the treatment. Gas exchange measurements were performed between 08:30 and 11:00 to avoid any potential midday depression in G_s values (eg. Tenhunen et al., 1984). The actual quantum efficiency of PSII (Genty et al., 1989) of the leaves was determined using a Hansatech FMS-2 fluorimeter (Hansatech, Norfolk, King's Lynn, UK). A Konica Minolta SPAD-502 (Konica Minolta, Tokyo, Japan) was used to gauge foliar chlorophyll concentration from an average of five readings per leaf (Marenco et al., 2009).

2.3. Leaf morphological analyses

Following gas exchange and chlorophyll fluorescence analysis, leaves were detached from the plant. The petiole was then cut at the base of the leaf, and the leaf was photographed using a Sony DSC-T99 14 megapixel camera. The computer program ImageJ (National Institutes of Health, Bethesda MD, USA) was used to determine leaf area from the digital images. After photography of the leaves, dental impression gel (President Light Body Material, Coltene, Altstätten, Switzerland) was used to create a negative impression of the abaxial leaf surface (Weyers and Lawson, 1985; Stein et al., 2024). *Ginkgo biloba* is hypostomatous

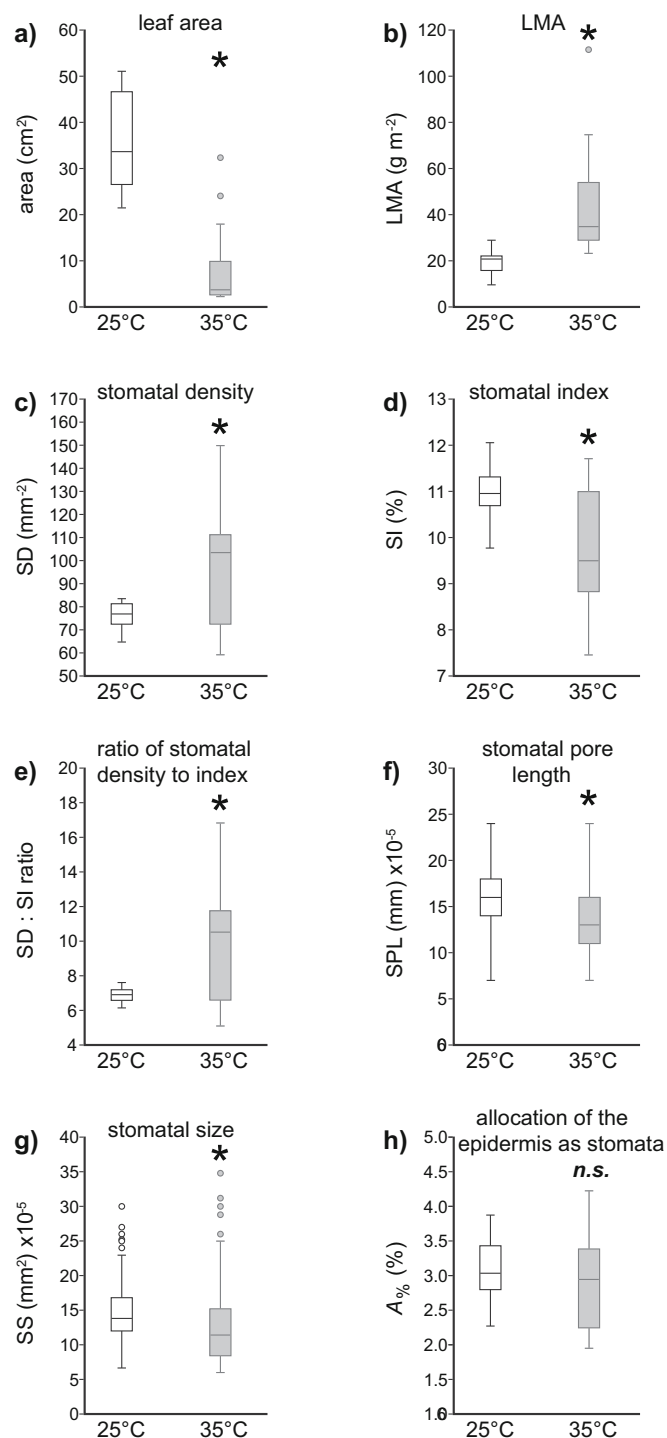


Fig. 1. Box plots illustrating the effect of growth at 25/20 °C (black lines, white fill) and 35/30 °C (dark grey lines, light grey fill) on macro- and micro-morphological parameters of *Ginkgo biloba* leaves: a) leaf area ($F_{1,51} = 120.746$; $P = 2.8 \times 10^{-15}$); b) leaf mass per area ($F_{1,51} = 41.512$; $P = 3.5 \times 10^{-8}$); c) stomatal density ($F_{1,51} = 16.772$; $P = 0.0002$); d) stomatal index ($F_{1,51} = 27.011$; $P = 3.6 \times 10^{-6}$); e) stomatal density to stomatal index ratio ($F_{1,50} = 24.056$; $P = 1.1 \times 10^{-5}$); f) stomatal pore length ($F_{1,719} = 83.086$; $P = 7.7 \times 10^{-19}$); g) stomatal size ($F_{1,720} = 28.942$; $P = 1.0 \times 10^{-7}$); h) allocation of the epidermis to gas exchange ($F_{1,50} = 2.836$; $P = 0.098$). The box signifies the distribution of the 25–75 % quartiles, the median is represented by a horizontal line within the box, horizontal bars either side of the box indicate minimum / maximum values, and circles indicate outliers.* indicates significant difference and n.s. indicates no significant difference between the 25 and 35 °C treatments at the 0.05 significance level using a one-way ANOVA.

(Haworth et al., 2018c), therefore stomatal analyses relate only to the lower abaxial surface. Thirty minutes after the application of the dental impression gel, the leaves were removed and oven dried at 70 °C for four days. When the weight of the *G. biloba* leaves had remained stable for at least two days, the weight of each leaf was recorded and used to determine leaf mass per area (LMA) (Bacon et al., 2016).

2.4. Stomatal morphological analyses

Nail varnish (Max Factor, Proctor and Gamble, Cincinnati OH, USA) was used to create ‘positive’ leaf impressions from the dental impression gel ‘negatives’. The nail varnish positive was then placed on a glass slide and imaged using a Leica DM2500 microscope with a Leica DFC 300FX camera (Leica Microsystems, Wetzlar, Germany). To measure stomatal density (SD) and stomatal index (SI), a 0.3×0.3 mm grid was superimposed over the image (avoiding areas of the leaf where veins were present) and the number of stomata and epidermal cells within the grid were counted from ten images per leaf (rarefaction analysis was used to identify a minimum number of 5 to 6 counts before the mean values of SD and SI stabilised) (Poole and Kürschner, 1999). The following dimensions were measured from 20 stomatal complexes per leaf: stomatal pore length (SPL); guard cell length (GCL), and; guard cell width (GCW). Stomatal complex size was calculated as $GCL * (2 * GCW)$ following Franks and Beerling (2009). Stomatal pore area (SPA) was calculated as the area of an ellipse where stomatal pore width is considered to be 0.5 SPL at maximum stomatal opening (Haworth et al., 2015). The proportion of the epidermis available for gas exchange (A_e) was calculated

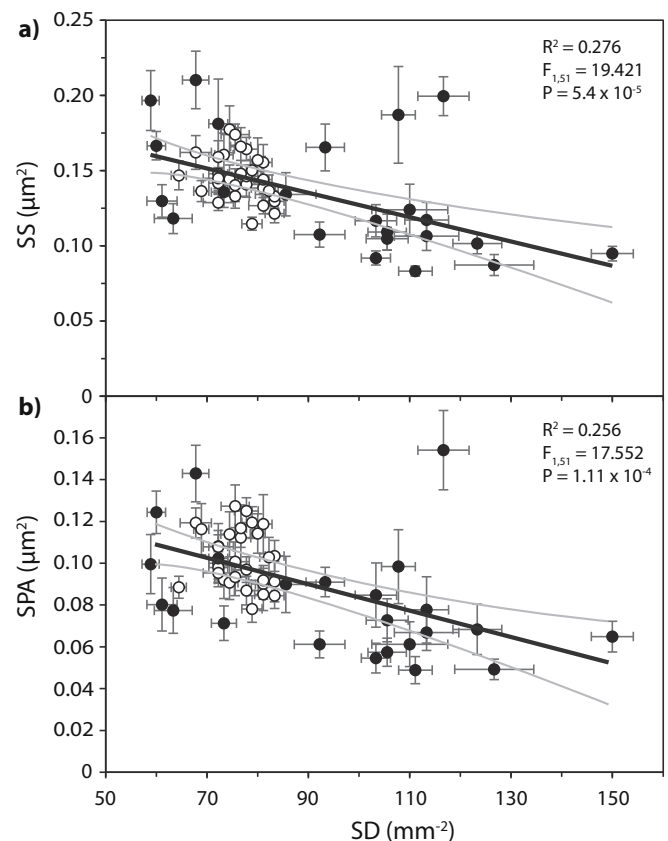


Fig. 2. The relationship between stomatal density (SD) with (a) stomatal size (SS) and (b) stomatal pore area (SPA) of *Ginkgo biloba* leaves grown in the 25 °C (white fill symbols) and 35 °C (black fill symbols) treatments. The black line indicates the line of best fit and the two grey lines either side indicate the 95 % confidence intervals of the mean. Linear regression was used to assess the significance of any relationships – data given in right-hand corner of each panel. Error bars indicate one standard error either side of the mean.

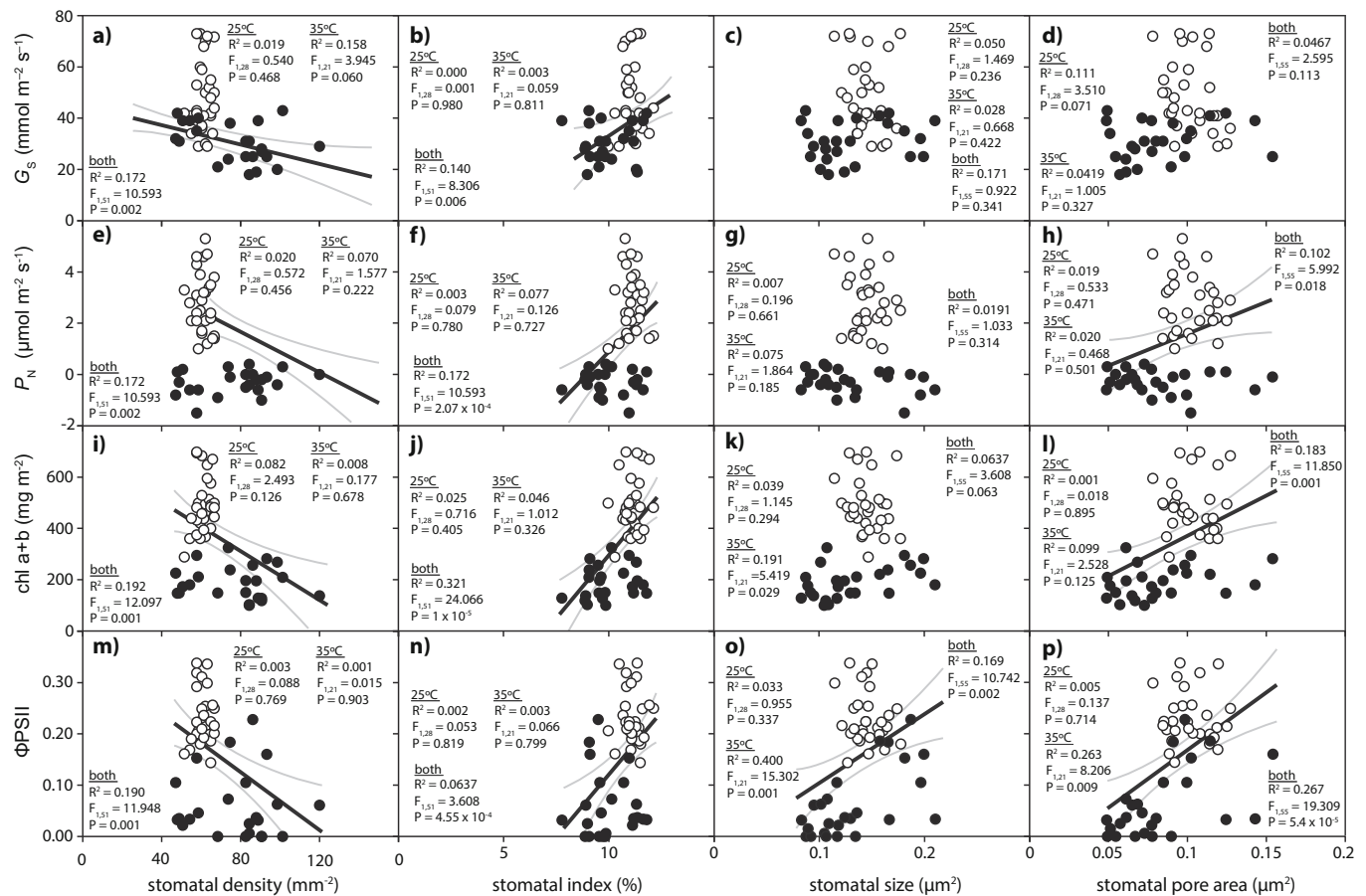


Fig. 3. Relationships between measured leaf-level stomatal conductance (G_s), photosynthesis (P_N), leaf chlorophyll content (chl a + b), and the actual quantum efficiency of photosystem II (Φ PSII) with leaf micro-morphological characters of stomatal density (a, e, i, m), stomatal index (b, f, j, n), stomatal size (c, g, k, o), and stomatal pore area (d, h, l, p) of *Ginkgo biloba* leaves grown in the 25 °C (white fill symbols) and 35 °C (black fill symbols) treatments. The black line indicates the line of best fit and the two grey lines either side indicate the 95 % confidence intervals of the mean. Linear regression was used to assess the significance of any relationships – in cases where a significant relationship was observed in the dataset where both temperature treatments were grouped together the regression line was drawn.

from SPA at theoretical maximum stomatal opening. Guard cells were assumed to be circular in transverse cross-section, allowing GCW to be used for guard cell depth in the calculation of theoretical maximum G_s , with stomatal pores treated as an empty cylinder using the formulae of Cowan (1978). The stomatal parameters of the *G. biloba* leaves were used to estimate the $[CO_2]$ in which they developed using the SI- $[CO_2]$ transfer function of Royer et al. (2001), and the ‘recent calibration’ of McElwain and Chaloner (1996) using an SI value of 12.43 % for *G. biloba* grown under $[CO_2]$ of 380 $\mu mol mol^{-1}$ in a broadly comparable controlled environment chamber setting (Haworth et al., 2013).

3. Results

Heat stress affected leaf macro-morphology with a 79.2 % decrease in leaf area (Fig. 1a) and a 66.1 % increase in LMA (Fig. 1b) observed in the 35 °C treatment. The SD of leaves grown in the 35 °C treatment was on average 26.2 % higher than leaves from the 25 °C treatment with a wider range of SD values recorded at the higher temperature (Fig. 1c). The mean SI of *G. biloba* leaves in the 25 °C treatment was 11.1 %, while the 35 °C treatment was 9.9 %, indicative of a 10.1 % decrease in stomatal initiation at the higher temperature (Fig. 1d). Stomatal pore length and SS were respectively 13.7 and 12.6 % lower in the 35 °C treatment. Despite the differences in SD and SS of *G. biloba* leaves grown in the two temperature regimes, there was no significant difference in $A_{\%}$ of the leaves developed in the 25 and 35 °C treatments (Fig. 1h). Stomatal size and SD showed significant negative relationships across

G. biloba leaves grown in the two temperature regimes (Fig. 2).

Stomatal conductance (Fig. 3a) and P_N (Fig. 3e) rates were negatively correlated with SD in *G. biloba* leaves developed in the 25 and 35 °C treatments. Similar negative correlations were observed between SD with foliar chlorophyll content (Fig. 3i) and Φ PSII (Fig. 3m), with generally lower values observed in leaves from the 35 °C treatment. The rate of stomatal initiation expressed by SI showed positive relationships to G_s (Fig. 3b) and P_N (Fig. 3f). Stomatal conductance was not significantly related to either SS (Fig. 3c) or SPA (Fig. 3d) at maximal stomatal opening. Photosynthesis was significantly but weakly ($R^2 = 0.102$) correlated to SPA at theoretical maximum stomatal opening in *G. biloba* leaves developed in the two temperature regimes (Fig. 3h). This is supported by positive correlations between maximum SPA with foliar chlorophyll content (Fig. 3l) and Φ PSII (Fig. 3p), where generally lower values of chlorophyll and Φ PSII were observed in the 35 °C treatment.

Calculated theoretical maximum G_s showed no significant correlation with observed G_s from gas exchange analysis (Fig. 4). Furthermore, no significant correlations between theoretical maximum and observed G_s were observed when the regression analysis was performed on leaves from each temperature regime in isolation. The observed G_s showed a significant negative relationship with SD in the *G. biloba* leaves, with the leaves from the 35 °C treatment showing generally higher SD and lower G_s values than their counterparts from the 25 °C treatment (Fig. 5a). No significant relationship was found between observed G_s and $A_{\%}$ (Fig. 5b). In contrast, theoretical maximum G_s showed significant positive relationships with SD (Fig. 5c) and $A_{\%}$ (Fig. 5d). It is worth noting

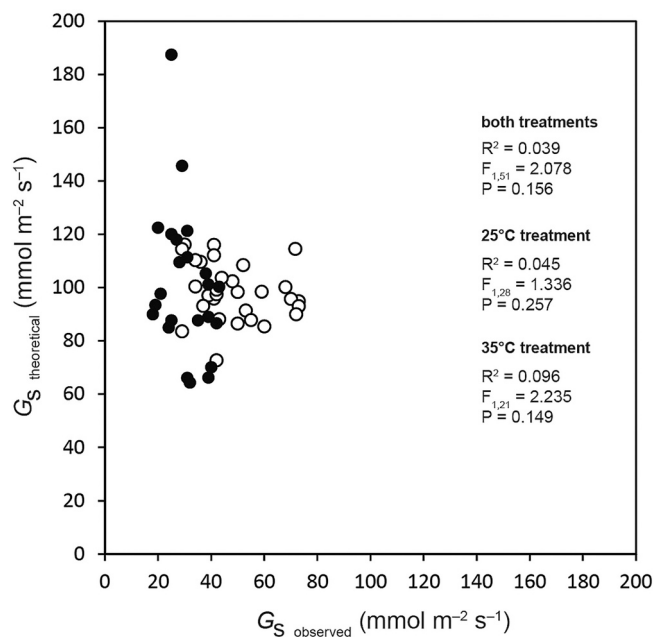


Fig. 4. Theoretical maximum stomatal conductance (G_s theoretical) calculated from stomatal morphological parameters versus observed stomatal conductance (G_s observed) measured using leaf gas exchange of *Ginkgo biloba* leaves grown in the 25 °C (white fill symbols) and 35 °C (black fill symbols) treatments. Linear regression was used to assess the significance of any relationships – data given in right-hand corner of the panel.

that parameters such as SD used to calculate theoretical maximum G_s also used to determine $A_{\%}$, resulting in self-correlation in Fig. 5d.

Despite growing in atmospheres with identical levels of ambient atmospheric $[CO_2]$, when the stomatal parameters of the *G. biloba* leaves from the two temperature regimes were used to gauge the $[CO_2]$ in which the leaves had developed, they produced statistically different results (Fig. 6). The NLE method with the Recent Calibration produced a 12.4 % higher estimate of $[CO_2]$ in leaves from the 35 °C treatment. The transfer function approach resulted in a 7.8 % higher estimate of $[CO_2]$ in leaves from the 35 °C temperature treatment. The NLE method using the Recent Calibration (that assumes a 1:1 stomatal ratio between the plant serving as the $[CO_2]$ proxy and its NLE) produced estimates of $[CO_2]$ above ambient levels (4.8–17.8 % higher), while the transfer function resulted in sub-ambient $[CO_2]$ estimates (17.8 to 23.6 % lower).

4. Discussion

The viability of the ‘stomatal method’ as a proxy of palaeo-atmospheric $[CO_2]$ is dependent upon stomatal morphology and initiation being affected only, or predominantly, by the $[CO_2]$ in which the leaf developed (Chaloner and Creber, 1990; McElwain and Chaloner, 1996; Beerling and Royer, 2002). The robustness of this assumption is of particular significance for leaves developed and deposited during episodes of global warming in Earth history, where the putative role of palaeo- $[CO_2]$ as a driver of global temperatures is relevant to predictions of future climate change in response to anthropogenic emissions of CO_2 (eg. McElwain et al., 1999; Retallack, 2002; Smith et al., 2010; Steinhilber et al., 2011). The results of the present study suggest that at high temperatures, where heat stress affects physiological function (Fig. 3) and leaf development (Haworth et al., 2018a), stomatal proxies of $[CO_2]$ based on Ginkgoales may become less reliable (Figs. 4 and 6).

Heat stress affected both leaf and stomatal development in the 35 °C treatment (Fig. 1). Physiological function was also impaired in the higher temperature treatment with lower P_N accompanied by reduced

foliar chlorophyll and quantum efficiency of PSII (Fig. 3) (Killi et al., 2017). One possible cause of reduced stomatal initiation in the 35 °C treatment (Fig. 6b) might be higher $[CO_2]$ in the sub-stomatal internal leaf air-space (Fig. 6a) inducing signals from mature to developing leaves to reduce stomatal initiation (Lake et al., 2001). The greater SD and smaller SS at the higher temperature coincided with reduced leaf expansion. Similar increases in SD with lower leaf area are observed in *Arabidopsis thaliana* (Clauw et al., 2015) and *Cannabis sativa* subject to water deficit (Haworth et al., 2024). The co-ordination of SS and SD in *G. biloba* from both temperature regimes is consistent with both allometric spatial scaling of SS and SD (Haworth et al., 2023) and a functional purpose, as high densities of small stomata are considered to facilitate the diffusional uptake of CO_2 (Franks and Beerling, 2009; Assouline and Or, 2013). The similarity in $A_{\%}$ values in the *G. biloba* leaves from the two treatments (Fig. 1h) may reflect an adaptation to enable leaves to maintain leaf gas exchange during a subsequent amelioration of temperature. Heat stress imposes significant leaf construction costs to the development of *G. biloba* leaves. These costs are particularly evident on an area basis, having implications for the light harvesting potential required to drive P_N (Haworth et al., 2018a). The maintenance of the capacity for photosynthetic gain over the course of the lifespan of the leaf may reflect an adaptive advantage in economic terms.

Photosynthesis is often positively correlated to both measured G_s (Körner et al., 1979) and $A_{\%}$ (Haworth et al., 2018c). Under conditions of heat stress, the positive association of P_N with measured G_s or potential gas exchange derived from morphological stomatal parameters was not present (Fig. 5). The effect of heat stress on the biochemical efficiency of photosynthetic CO_2 assimilation (Berry and Björkman, 1980; Feller et al., 1998; Crafts-Brandner and Salvucci, 2000) may have led to a disassociation between P_N and potential leaf gas exchange in *G. biloba* (Fig. 3). The replication of such an effect of heat stress would have had significant negative implications for the carbon and water use efficiencies of fossil plants during warming intervals in Earth history (Haworth et al., 2018a). It is noteworthy that *G. biloba* leaves from the 35 °C treatment also showed lower rates of G_s (Fig. 3), suggesting that an increase in transpirative cooling was not an available adaptive mechanism to mitigate the deleterious impact of heat stress (Killi et al., 2017). At high temperatures some plants increase G_s (Heath and Meidner, 1957; Raschke, 1970; Schulze et al., 1975), potentially resulting in enhanced transpirative cooling of leaves (Jones, 1999), whereas *Olea europaea* (Haworth et al., 2018b) and *Populus × euramericana* (Fares et al., 2011) exhibited a similar reductions in G_s to *G. biloba*, possibly as an adaptation to prevent excessive water-loss and hydraulic cavitation of xylem vessels during extreme transpirative demand.

The positive correlation between observed G_s measured using leaf gas exchange and theoretical maximum G_s (eg. McElwain et al., 2016) forms a major component of leaf gas exchange models of palaeo- $[CO_2]$ based on stomatal morphological parameters (Konrad et al., 2008; Grein et al., 2011; Franks et al., 2014; Roth-Nebelsick et al., 2014). The pronounced reduction of G_s measured in the 35 °C treatment resulted in a significant negative correlation between observed G_s and SD (Fig. 5a), while theoretical maximum G_s showed a positive relationship to SD (Fig. 5c) and $A_{\%}$ (Fig. 5d). The uncoupling of observed G_s from stomatal parameters of *G. biloba* under heat stress (reflected in the lack of a significant relationship between observed and theoretical G_s – Fig. 4) would undermine the utility of leaf gas exchange models of palaeo- $[CO_2]$ from Ginkgoales during intervals marked by pronounced temperatures inducing floral heat stress. This result provides empirical evidence to support sensitivity testing of diffusion based models at high temperature (Konrad et al., 2021).

Heat stress in the 35 °C treatment induced a significant reduction in stomatal initiation in *G. biloba* (Fig. 1d and 6b). If replicated in the fossil record, a heat stress induced reduction in SI would be construed to reflect an increase in palaeo- $[CO_2]$. This result may serve as a note of caution in the efficacy of stomatal palaeo- $[CO_2]$ reconstructions during

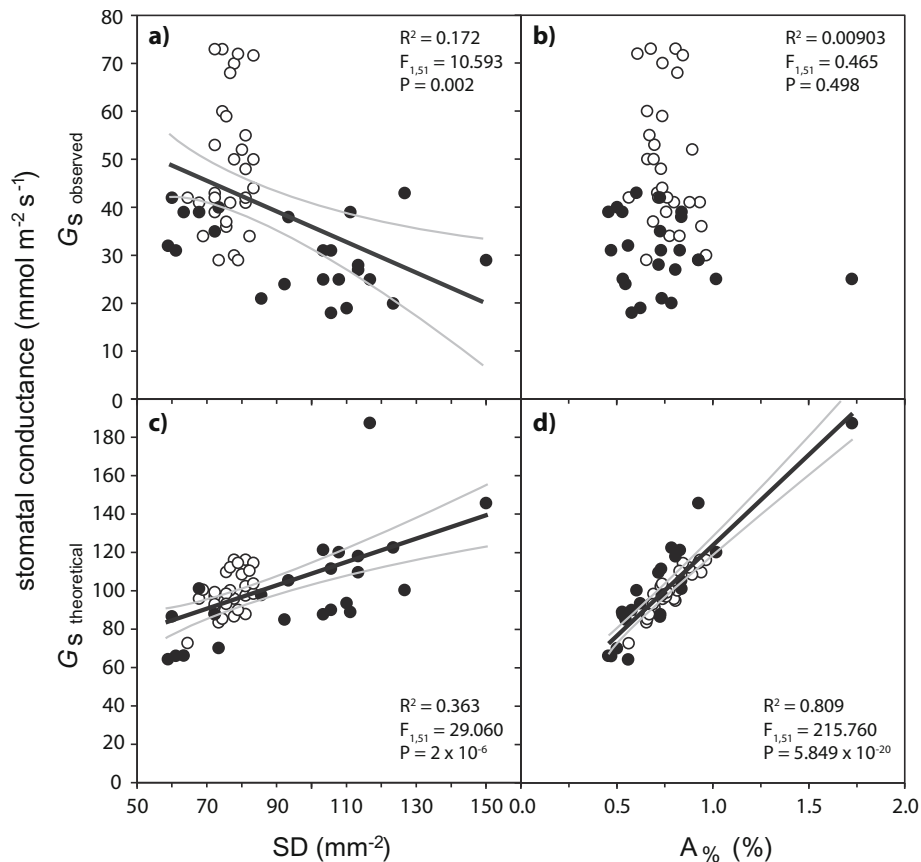


Fig. 5. Relationships between theoretical and observed stomatal conductance with (a, c) stomatal density (SD) and (b, d) the proportion of the epidermis allocated to gas exchange ($A\%$) of *Ginkgo biloba* leaves grown in the 25 °C (white fill symbols) and 35 °C (black fill symbols) treatments. The black line indicates the line of best fit and the two grey lines either side indicate the 95 % confidence intervals of the mean. Linear regression was used to assess the significance of any relationships – data given in right-hand corner of each panel.

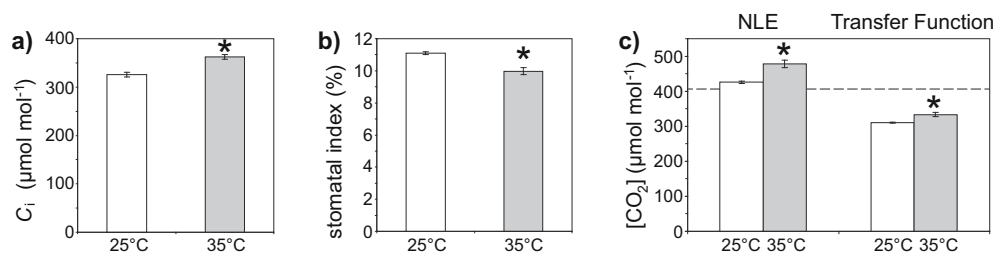


Fig. 6. The effect of growth in 20/25 °C (white fill) and 30/35 °C (grey fill) night/day temperature regimes on (a) the internal sub-stomatal concentration of carbon dioxide (C_i), and (b) the stomatal index of *Ginkgo biloba* leaves. Panel c indicates $[\text{CO}_2]$ reconstructions based on stomatal index values of the *G. biloba* leaves from the different temperature treatments using the nearest living equivalent approach of McElwain and Chaloner (1996) and transfer function approach of Royer et al. (2001) - the dashed horizontal line indicates the actual ambient $[\text{CO}_2]$ in which the *G. biloba* leaves developed. Error bars indicate one standard error either side of the mean. * indicates significant difference and n.s. indicates no significant difference between the 25 and 35 °C treatments at the 0.05 significance level using a one-way ANOVA.

intervals of pronounced global warming marked by ecological disruption to vegetation (eg. McElwain et al., 2007; Van de Schootbrugge et al., 2009; Mander et al., 2013). Nevertheless, inconsistency in the ratio of SD to SI in fossil plants through a stratigraphic section may be used as an indicator of potential plant stress influencing leaf expansion and stomatal initiation (Haworth et al., 2012). Fumigation with toxic sulphur dioxide (SO_2) induced reductions on leaf area (Bacon et al., 2013) and a concomitant increase in SD (Haworth et al., 2012) in plants without acquired resistance to SO_2 (Winner and Mooney, 1980; Ayazloo et al., 1982; Haworth et al., 2010). Heat stress produced pronounced increases in the SD to SI ratio (Fig. 1e) primarily driven by lower leaf expansion (Fig. 1a). Alongside other biogeochemical indicators of higher temperatures (eg. Weijers et al., 2007; de Bar et al., 2019), a shift in the SD to SI

ratio of fossil plants, such as that observed in Ginkgoales at the Astartekløft Triassic – Jurassic Boundary section (Haworth et al., 2012), may indicate heat stress effects on stomatal initiation and corresponding implications for the accurate reconstruction of palaeo- $[\text{CO}_2]$. The results of the present study are not inconsistent with the findings of Steinhorsdottir et al. (2022) who observed consistency in SI values in *G. biloba* cultivated in different climates. Our findings pertain to the specific effect of severe heat stress, at or beyond the tolerance limits of *G. biloba*, as evidenced by pronounced declines in the physiological capacity for photochemical light usage and CO_2 -assimilation (Fig. 3). The effect of heat stress on stomatal development of *G. biloba* may only become apparent at a threshold temperature. The observation of heat stress effects in a widely used NLE for stomatal palaeo- $[\text{CO}_2]$

reconstructions reinforces the need for further controlled environment studies of plant groups used as palaeo-[CO₂] stomatal proxies to refine and sensitivity test the stomatal method towards producing more robust reconstructions of palaeo-atmospheric [CO₂] over deep-time.

5. Conclusion

This study suggests that heat stress, as indicated by impaired photosynthetic function, affects stomatal initiation and development in *G. biloba*. *Ginkgo biloba* plants grown in the 35 °C treatment had lower SI values than their counterparts from the 25 °C treatment. If replicated in fossil Ginkgoales, this reduction in SI values would possibly be interpreted to indicate a rise in palaeo-[CO₂]. In light of the importance of *G. biloba* as a widely used NLE for palaeo-[CO₂] reconstructions over deep-time, this may have negative implication for the reliability of stomatal palaeo-[CO₂] reconstructions during global warming events in the geological record. However, consideration of biogeochemical indicators of heat stress, changes in macro- and micro-fossil records suggestive of vegetation species turnover, and shifts in the SD to SI ratio in stratigraphic contexts may be used to reliably indicate intervals where heat stress may have affected stomatal reconstructions of palaeo-[CO₂]. The use of similar controlled environment chamber experiments to examine the strength of the relationships between [CO₂] and stomatal development at different degrees of heat stress would be a valuable tool in evaluating the efficacy of the stomatal method in reconstructing palaeo-[CO₂] during intervals of rising global temperatures.

CRedit authorship contribution statement

Andrea Carli: Writing – review & editing, Investigation, Data curation. **Mauro Centritto:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Alessandro Materassi:** Writing – review & editing, Investigation. **Dilek Killi:** Writing – review & editing, Investigation, Data curation. **Giovanni Marino:** Writing – review & editing, Investigation. **Antonio Raschi:** Writing – review & editing, Resources, Project administration, Conceptualization. **Matthew Haworth:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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