A high-throughput method for measuring critical thermal limits of leaves by chlorophyll imaging fluorescence

Short title: Thermal limits and chlorophyll fluorescence

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One sentence summary:
There are species-specific effects of experimental conditions during the measurement of the critical thermal limits of leaf photosynthetic performance.

Author contribution statement:
PAA, KMG, AAC, and ABN designed the experiments. PAA, KMG, AAC performed the experiments and collected the data. PAA curated the data and performed the data analyses and visualisation. PAA, VFB, LAB, and ABN interpreted the results and wrote the manuscript with input from all authors.

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Abstract

Plant thermal tolerance is a crucial research area as the climate warms and extreme weather events become more frequent. We developed and tested a high-throughput method for measuring photosynthetic critical thermal limits at low (CT_{MIN}) and high (CT_{MAX}) temperatures to achieve pragmatic and robust measures of thermal tolerance limits using a Maxi-Imaging fluorimeter and a thermoelectric Peltier plate temperature ramping system. Leaves exposed to temperature extremes accumulate damage to photosystem II (PSII). Temperature-dependent changes in basal chlorophyll fluorescence (T-F_0) can be used to identify the critical temperature at which PSII is damaged. We examined how experimental conditions: wet *vs* dry surfaces for leaves and temperature ramp rate, affect CT_{MIN} and CT_{MAX} across four species. CT_{MAX} estimates were not different whether measured on wet or dry surfaces, but leaves were apparently less cold tolerant when on wet surfaces. Temperature ramp rate had a strong effect on both CT_{MAX} and CT_{MIN} that was species-specific. We discuss potential mechanisms for these results and recommend settings for researchers to use when measuring T-F_0. The system described and tested here allows high-throughput measurement of critical temperature thresholds of leaf photosynthetic performance for characterising plant function in response to thermal extremes.

Introduction

Understanding both vulnerability and tolerance of plants to thermal extremes is a priority for plant biology research as the Earth's climate continues to change, thereby exposing these sessile organisms to increased thermal stress (O'Sullivan et al., 2017; IPCC, 2018; Geange et al., under review). Thermal stress disrupts and inhibits physiological processes (Goraya et al., 2017), induces protective and repair mechanisms (Sung et al., 2003; Goh et al., 2012), leads to declines in plant performance, and threatens survival (Zinn et al., 2010; Bita and Gerats, 2013). Plant photosynthesis is highly sensitive to thermal stress and has distinct thermal limits beyond which tissue damage begins to accumulate (Neuner and Pramsohler, 2006). The temperature sensitivity of photosynthesis mostly derives from the thermally-dependent stability of protein-pigment complexes in the light harvesting complex II (LHCII) of photosystem II (PSII) of the thylakoid membrane of chloroplasts (Ilík et al., 2003), which is integral to the photosynthetic electron transport chain (Berry and Björkman, 1980; Mathur et al., 2014).

Chlorophyll fluorimetry has become a widely used tool for assessing the thermal limits of photosynthesis for both cold and heat tolerance (Geange et al., under review). Chlorophyll can dissipate absorbed light energy via photochemistry, or re-emit it as heat energy or fluorescence (Baker, 2008; Murchie and Lawson, 2013). A dark-adapted leaf exposed to a low-

intensity modulated measuring light, which does not induce electron transport, emits a minimal amount of chlorophyll-*a* fluorescence from LHCII, called F_0. Under more intense or actinic light, processes that are highly dynamic and sensitive to other factors but not well correlated with the viability of the photosynthetic tissue cannot be isolated from the measurement of the temperature dependence (thermal stability) of chlorophyll fluorescence. To assess the thermal stability limits of LHCII, plant ecophysiologists typically measure the temperature-dependent change in basal chlorophyll-*a* fluorescence (T-F_0) to determine the critical temperature threshold (T_{crit}), denoted by a sudden increased in F_0 at which PSII undergoes temperature-dependent conformational changes and begins to incur thermal damage (e.g., Schreiber and Berry, 1977; Berry and Björkman, 1980; Knight and Ackerly, 2002; Ilík et al., 2003; Hüve et al., 2006; Neuner and Pramsohler, 2006; O'Sullivan et al., 2013; O'Sullivan et al., 2017; Zhu et al., 2018). F_0 is a fluorescence parameter that can be measured rapidly and continuously throughout heating or cooling in darkness, without the need of a saturating pulse and re-dark adaptation as for F_V/F_M measurements that are commonly used to detect photosynthetic damage.

One critique of T-F_0 measurements and T_{crit} determination is that they are conducted on detached leaves. Detaching leaves to expose them to a precisely controlled and measured thermal surface is usually, but not always, a necessary component of this trait measurement. Leaf detachment can affect leaf hydration and fluorescence through reduced PSII activity, ionic leakage, and oxidations compared to attached leaves (Potvin, 1985; Smillie et al., 1987). Leaf dehydration could be problematic for certain species if leaves are sampled long before they are assessed for T_{crit} or if they are measured as leaf sections or discs. To avoid dehydration during the T-F_0 measurement, a wet surface (e.g., damp paper surface as in Knight and Ackerly (2002)) could physically impair evaporation by saturating the atmosphere surrounding the leaf. However, it is not clear whether a wet surface interferes with the T-F_0 measurement or how it might affect the T_{crit} value compared to using a dry surface.

A great advantage of using temperature-dependent changes in chlorophyll fluorescence and a thermoelectric plate is that both cold and heat tolerance of leaves can be measured with much of the same equipment. However, the protocol may need to be altered slightly because cold transitions in nature occur much more slowly than heat transitions, which may induce different mechanisms in response to thermal stress. For example, leaf temperature can rapidly increase during a lull in wind speed, far exceeding ambient temperature on a hot and sunny day (Vogel, 2009; Leigh et al., 2012). On a cold frosty night, even considering air temperature stratification, the rate of leaf temperature cooling rarely exceeds 5°C h^{-1}, especially below freezing (Sakai and Larcher, 1987). Therefore, the ‘standard’ protocols for measuring T_{crit}

typically ramp temperature changes much faster for heat tolerance than for cold tolerance. While this approach is justified by rates observed in natural systems, the first published application of the T-F_0 technique (Schreiber and Berry, 1977) used an apparently arbitrary 'slow' heating rate of 1°C min^{-1} (i.e., 60°C h^{-1}). Subsequently, while many studies followed suit, a vast range of ramp rates have been applied (see Table S1), often with little justification. We have known for decades that different rates of heating and cooling can affect the T-F_0 curve and shift the T_{crit} value by at least 2°C (Bilger et al., 1984; Frolec et al., 2008). Therefore, studies employing T-F_0 methods for measuring thermal tolerance limits that use different temperature ramp rates might not be directly comparable, even within a given species. Further, it is reasonable to expect that plant species might exhibit different responses to variation in methodology.

Here, we present a practical, high-throughput method for measuring T_{crit} with a Pulse Amplitude Modulated (PAM) chlorophyll fluorescence imaging system that measures F_0 in real time as temperature is ramped up or down to a thermal extreme using a thermoelectric Peltier plate. We then investigate variations of easily controllable variables of the standard experimental protocol that could affect thermal tolerance estimates. We sought to determine the effects of wet *vs* dry surface and temperature ramp rate on T_{crit} estimates for both the heat tolerance limit (hereafter referred to as critical maximum temperature; CT_{MAX}) and the cold tolerance limit (hereafter referred to as critical minimum temperature; CT_{MIN}) of leaf thermal stability of species with different growth and leaf forms. By comparing among these species, we also determined whether the effects of the two experimental variables could be generalised for different types of plants that might originate from glasshouse or field conditions. In doing so, we advise researchers on what we consider to be a pragmatic approach to measuring leaf thermal tolerance using chlorophyll imaging fluorescence, at a time when improved understanding of plant tolerance to thermal extremes is needed for cultivated and wild species alike.

Results

Overview

The Peltier plate and chlorophyll fluorescence Maxi-Imaging-PAM system allows us to measure T-F_0 (Fig. 1) on many leaves simultaneously. In these experiments, we measured up to 30 whole leaf samples in a single experimental run, which could take as little as 90 minutes including dark adaptation, leaf set up on the surface, and the temperature ramp (at 60°C h^{-1}). The Peltier plate can easily accommodate a much greater number of smaller leaves, leaf discs, or leaf sections for even higher throughput phenotyping (Fig. S1).

Surface wetness experiment: effect of wet *vs* dry surface for leaves on CT_{MIN} and CT_{MAX}

The effect of water saturating the filter paper was clearly apparent for T_{crit} value estimates for CT_{MIN} (Fig. 2A) but not CT_{MAX} (Fig. 2B). For all species combined and when the three species were analysed separately, CT_{MIN} values were significantly and consistently less negative (less cold tolerant) for leaves on wet surfaces than on dry ones, by 3–4°C (Table 1, S2, Fig. 2A). Variation in CT_{MIN} was independent of the initial F_V/F_M of leaves. The CT_{MAX} of leaves with a wet paper surface did not differ significantly from dry ones both among and within species (all $p > 0.2$; Table 1, S2, Fig. 2B), although the three species had different CT_{MAX} estimates. Leaves with higher F_V/F_M had higher CT_{MAX} for *W. ceracea*.

Surface wetness × ramp rate experiment: effects on CT_{MIN} and *NT*

CT_{MIN} of leaves of all species was higher on a wet surface and generally lower at faster ramp rates compared to leaves on a dry surface at slower ramp rate (Table S3). However, the interaction between surface wetness and ramp rate never had a significant effect on CT_{MIN}; leaves on a wet surface had a consistently higher CT_{MIN} than those on a dry surface at both 15 and 60°C h^{-1}. A small subset of 17 leaves could be used to test whether surface wetness and ramp rates affected *NT*, however, due to this low sample size, we opted not to formally analyse these data, but present descriptive findings in Fig. S2. *NT* of leaves measured on a wet surface occurred at higher temperatures (around -7°C) independently of ramp speed, however *NT* occurred at lower temperatures on leaves on a dry surface, and perhaps slightly lower on leaves exposed to a faster ramp (Fig. S2). *NT* generally occurred at temperatures 2-4°C higher than CT_{MIN}, and the mean difference between CT_{MIN} and *NT* was 1°C lower on a wet surface compared to a dry surface (Fig. S2).

Ramp rate experiment: effect of temperature ramp rate on CT_{MIN} and CT_{MAX}

Varying temperature ramp rate affected the estimate of T_{crit} for CT_{MIN} and CT_{MAX} considerably, however each species responded differently. For CT_{MIN}, slow ramp rates (< 10°C h^{-1}) are standard practice and here we used 3°C h^{-1} as the reference category. We found no significant differences between 3, 6, 15, or 30°C h^{-1} ramp rates overall, but when the plate was ramped at the faster speeds, the CT_{MIN} values became very different to the slower ramp rates. At 60 and 240°C h^{-1} CT_{MIN} was significantly lower relative to 3°C h^{-1} for *M. citrina* and *E. rubra* (Table 2, S4). For *M. citrina*, the values shifted depending on ramp rate, but with no clear pattern (Fig. 3A). In contrast, *E. rubra* had stable CT_{MIN} values for 3, 6, and 15°C h^{-1} and more negative values as ramp rate increased to 30, 60, and 240°C h^{-1} (Table 2, S4, Fig. 3A). CT_{MIN} for *W.*

ceracea was similar across most ramp rates and was only significantly different from when the ramp rate was 30°C h^{-1} (Table 2, S4). Variation in CT_{MIN} was independent of the initial F_V/F_M of leaves.

CT_{MAX} is typically measured at 60°C h^{-1}, so this rate was used as a reference against which all other ramp rates were compared. CT_{MAX} was highly dependent on temperature ramp rate, where rates slower than 60°C h^{-1} produced significantly lower CT_{MAX} estimates, except for 6°C h^{-1}. Ramp rates higher than 60°C h^{-1} resulted in higher CT_{MAX} estimates, significantly so for 240°C h^{-1} but not 120°C h^{-1} (Table 2, S5). However, stark species-specific responses were evident. CT_{MAX} in *M. citrina* was very low at ramp rates of 6 and 15°C h^{-1} and increased significantly and consistently with faster ramp rates: only 45 and 60°C h^{-1} yielded similar CT_{MAX} values (Table 2, S5, Fig. 3B). In contrast, CT_{MAX} in *E. rubra* was higher at the slowest rate (although the effect was marginal) compared to 60°C h^{-1} but significantly lower at 30 and 45°C h^{-1} and not different from 120 and 240°C h^{-1} (Table 2, S5, Fig. 3B). Similarly, *W. ceracea* had significantly higher CT_{MAX} values at 6°C h^{-1}, but also at 120 and 240°C h^{-1}. Only 45 and 60°C h^{-1} produced CT_{MAX} values for *W. ceracea* that were not significantly different (Table 2, S5, Fig. 3B). In all analyses except *E. rubra* individually, F_V/F_M had a significant positive relationship with CT_{MAX}.

Ramp rate experiment: predicted thermal limits as a function of ramp rate

We then modelled predicted CT_{MIN} and CT_{MAX} values against ramp rate as a continuous variable using a quadratic function to visualise the interspecific differences in response to different ramp rates when measuring thermal tolerance (Fig. 4). The difference between 60 and 240°C h^{-1} introduced extreme uncertainty in the predicted CT_{MIN} for *M. citrina*, so the 240°C h^{-1} rate was removed from the visualisation. The shape of each species' CT_{MIN} and CT_{MAX} response to ramp rate were clearly distinct from one another and only *E. rubra* had a relatively stable predicted CT_{MAX} value across all measured ramp rates. The variance tends to increase with faster ramp speeds for CT_{MIN}, but the pattern is less clear for CT_{MAX}.

Discussion

We sought to develop a reliable, high-throughput method for assessing thermal tolerance of the photosynthetic apparatus. Many methods are used for measuring plant thermal tolerance, but such variation has potential consequences for generating reasonable interpretations and interspecific comparisons. Often, the rationale behind a published method is unclear and the impacts of small methodological differences are difficult to assess. To address this, we have

demonstrated a method for measuring both cold and heat tolerance limits of leaves using a thermoelectric plate and chlorophyll imaging fluorescence. In line with previous applications of this technique, we provide evidence for the effects of controllable experimental variables on estimates of CT_{MIN} and CT_{MAX}. We quantify the significant effects of measurement conditions and show that using a wet *vs* dry surface for measuring CT_{MIN} and that variation in temperature ramp rates leads to substantial differences in CT_{MIN} and CT_{MAX}. We aimed to develop a practical method that maximises informative value and minimises experimental noise among samples. In the case of ramp rate, there is high species specificity. Below we outline potential mechanistic explanations for our findings along with testable hypotheses, and then propose best practices for measuring the thermal tolerance limits of leaves.

Pros and cons of the T-F_0 Peltier plate-Maxi-Imaging fluorimeter method

Measuring the temperature-dependent change in basal chlorophyll fluorescence is one of several potential methods that researchers can use to quantify the thermal limits of the photosynthetic apparatus stability (Ilík et al., 2003). The method that we present here offers improvements over earlier and alternative versions that use bulky water baths or freezing chambers, or smaller capacity Peltier plates (e.g., Schreiber and Berry, 1977; Braun et al., 2002; Knight and Ackerly, 2002; Neuner and Pramsohler, 2006), and adds several key features. The Peltier plate-Maxi-Imaging fluorimeter system is relatively compact and transportable for field applications when provided with a continuous power source. It offers precise temperature control and high versatility by being programmable for both cooling and heating rapidly at set ramp rates. It can be programmed for stepwise temperature treatments or non-linear temperature programs, or temperature shock treatments depending on the desired application. Furthermore, the T-F_0 curve allows for the calculation of other parameters (e.g., Knight and Ackerly, 2002), including the temperatures at 50% or 100% of relative F_0 (T_{50} and T_{max}, respectively) and ice nucleation temperatures (*NT*) for cold tolerance assays if each leaf sample has a thermocouple attached to it (e.g., Briceño et al., 2014). When using detached leaves or leaf discs, the potential throughput of the system is substantial (Fig. S1). The 120 × 90 mm optimal imaging area on the Peltier plate can fit > 100 leaf discs or small leaf samples up to 1 cm^2 or > 30 samples that are up to 2cm^2 each, thus throughput is mostly constrained by sampling and setting up that many leaves.

As with any laboratory equipment, there are limitations to the Peltier plate-Maxi-Imaging fluorimeter system. Unlike freezing chambers, this system does not allow for whole-plant measurements. There is some software modification required for controlling the ramps using the Peltier plate system, although newer temperature controllers and software revisions

than those used here are now available. The Peltier plate-Maxi-Imaging fluorimeter system is a versatile phenotyping tool for thermal tolerance, ecophysiology, and photosynthesis research. Below, we discuss the results of testing the system with wet and dry filter paper as surfaces and the effects of ramp rates.

A dry surface avoids experimental artefacts

Using wet filter paper as a surface for the leaf samples significantly reduced the apparent measured cold tolerance but had no effect on heat tolerance. Wet filter paper was initially tested to attempt to avoid leaf dehydration by providing a saturating atmosphere, preventing leaf evapotranspiration. In our cold tolerance assay, freezing of the water in the wet filter paper most likely began propagating ice from outside the leaf into the apoplastic space, thereby freezing the apoplast in the leaf tissue at higher temperatures than leaves on the dry surface. When radiative frost occurs, air humidity condenses on the leaf surface, resulting in a wet leaf surface that may induce heterogenous extrinsic nucleation in natural frosts (Pearce, 2001). Thus, the wet filter paper surface acted as an extrinsic ice nucleator and likely prevented the leaves from supercooling (Sakai and Larcher, 1987; Pearce, 2001; Larcher, 2003). Our exploratory test between wet and dry surfaces at different ramp rates demonstrated that on a dry filter paper surface, leaves appeared to supercool 2-4°C below those leaves on a wet surface. *NT* occurred earlier and at temperatures closer to CT_{MIN} on the wet surface and was more variable in comparison to leaves on a dry surface. Although this supercooling phenomenon requires further targeted investigation in future, our initial tests suggest that a wet surface induces earlier ice formation and propagation at warmer temperatures and hence reduces leaf supercooling capacity, and that supercooling capacity might be exacerbated by faster ramp rates.

The initial water status of leaf samples is still crucial, as water-stressed leaves can have compromised (Verslues et al., 2006) or even enhanced stress tolerance (Havaux, 1992). Therefore, we recommend that detached leaves should be transported in a manner that maintains leaf water content after sampling (e.g., sealing leaves with plastic film wrap, using damp paper towel, or cut stems placed in water) so that leaves are either maintained at collection conditions or fully hydrated at the start of the thermal tolerance assay.

Maximising throughput without compromising results

A wide range of temperature ramp rates have been used in previous studies of thermal tolerance limits (Table S1). We have demonstrated that temperature ramp rate strongly influences both CT_{MIN} and CT_{MAX} values with varying magnitude and complex patterns for different species.

Indeed, we saw such strong species-specific responses to different ramp rates (particularly for heat tolerance) that if one were to measure the CT_{MAX} for three species measured at the same ramp rate of 45°C h^{-1}, they would conclude that all the species have identical heat tolerance, yet the same experiment conducted with a ramp rate of 6°C h^{-1} and 240°C h^{-1} would result in entirely different, and opposing, conclusions. For comparative studies that measure species with different leaf morphology, physiology, and biochemical constituents, it is crucial that we clarify and refine what physiological event(s) we aim to characterise with the T-F_0 approach. From a practical standpoint, our aim was to identify the fastest ramp rates that would allow repeatable, interpretable measures of T_{crit}.

Temperature ramp rates will determine the potential for activation and extent of the upregulation of physiological processes and protective mechanisms within the leaf when approaching thermal extremes (Bilger et al., 1984; Frolec et al., 2008). The rise in F_0 during a measure of CT_{MAX} results from the blocking of electron transport in PSII and serves as an indicator of the thermal stability of the thylakoid membrane (Havaux et al., 1988; Nauš et al., 1992). If leaf samples are heated only up to the temperature of the initial rise in F_0, CT_{MAX}, and then cooled, it is possible that membrane damage can be reversed (Yamane et al., 1997; Frolec et al., 2008). However, irreversible damage to PSII through physiological changes to the photosynthetic apparatus and then physical membrane separation (i.e., denaturation) is correlated with the continued rapid rise and maxima of F_0 (Terzaghi et al., 1989; Frolec et al., 2008). Specifically, the first peak in F_0 shortly after CT_{MAX} and between 40-50°C is due to irreversible inactivation of PSII and the secondary F_0 peak between 55-60°C originates from the denaturing of chlorophyll-containing protein complexes (Ilík et al., 2003). Leaves can reduce the photochemical and oxidative damage induced by heat stress by thermal dissipation of excessive excitation energy to maintain PSII in an oxidative state, and by upregulating heat shock proteins and antioxidant activity (Allakhverdiev et al., 2008; Silva et al., 2010). Changes to the lipid composition of the thylakoid membrane reduces the fluidity of the membrane thereby being more stable at high temperatures (Allakhverdiev et al., 2008). The upregulation of these protective mechanisms of PSII can occur relatively quickly, sometimes < 1 h of heat stress (Havaux, 1993), thus how protected the leaf is against thermal damage will depend on the temperature ramp rate.

For cold tolerance assays, ramp rates likely modify the dynamic and primary site of ice nucleation. Intrinsic ice nucleation may lead to ice formation in the xylem (Hacker and Neuner, 2007), while extrinsic nucleation occurs at the leaf epidermis (Pearce and Ashworth, 1992). Rates of cooling may also influence supercooling capacity; usually faster cooling (within the

range of this study) increases supercooling capacity (Gokhale, 1965). Despite most freezing studies using ramp rates that are more reminiscent of natural freezing rates (≤ 5°C h^{-1}), we did not find a clear difference among CT_{MIN} values at ramp rates of 3, 6, and 15°C h^{-1}. We hypothesise that ramping the temperature relatively slowly (e.g., ≤ 15°C h^{-1}) could allow the cell to adjust osmotically and partially counterbalance the reduced water potential of the frozen apoplast restricting cell dehydration, which would be avoided at faster ramping speeds. Thus, the consideration for the freezing tolerance ramp rates becomes a question of what is the greatest ramp rate that allows more realistic osmotic adjustments within the leaf.

For *W. ceracea* and *E. rubra*, increasing temperature slowly (< 30°C h^{-1}) appears to allow time for induction of protective mechanisms such that slower ramp speeds result in higher CT_{MAX} values. Conversely, ramping temperature more quickly (30–60°C h^{-1}) prevents membranes from inducing heat-hardening or for antioxidants to be upregulated and take effect, such that measured heat tolerance is relatively stable at these ramp speeds. Our results indicate that beyond a rate of 60°C h^{-1}, the increase in F_0 occurs more slowly than the temperature increase and the temperature of the leaf samples (as measured by thermocouples) also lags significantly behind the temperature of the Peltier plate, thus the CT_{MAX} may be overestimated (Fig. 3B). Hence, using the thermistor (plate) temperature will overestimate the temperature of the leaf, and therefore, its tolerance. Furthermore, the faster that the plate temperature is changed, the more potential variation among leaf temperatures. We acknowledge that the method could be improved by using individual thermocouples for each leaf sample, particularly for cold tolerance to measure ice nucleation temperature (*NT*), however, we have verified that there is minimal variation (±1°C) across the Peltier plate surface.

The species specificity of the ramp rate dependence of CT_{MAX} was striking, particularly in the case of *M. citrina*. A slow ramp rate of 6°C h^{-1} results in a very low estimate for CT_{MAX} of only 36°C, which suggests that the heat tolerance of this species is poor, yet at ramp rates ≥ 30°C h^{-1}, this species is apparently as or more heat tolerant than the other species. Slow ramp rates mean that the leaves are slow to reach more stressful temperatures, but also that they are held at these temperatures for longer periods of time. We hypothesise that the lower heat tolerance at slow ramp rates could be due to leaf water being tightly bound and preventing cooling via transpiration or the heated leaf oils being unable to volatilise, thereby destabilising membranes and effectively ‘slow-cooking’ the leaf. For this species, the higher ramp rates are therefore likely more indicative of photosynthetic thermal tolerance.

The T-F_0 method is a rapid measurement compared to other F_V/F_M-based assessments of thermal tolerance. Determining the temperature at which 50% of the potential thermal damage

(lethal temperature) to the plant tissue occurs (LT_{50}) is a common but very time-consuming technique that also requires more plant material. Different individual leaves are ramped to and held at set temperatures for 1-3 h, and then F_V/F_M is measured over 1-24 h post-thermal exposure to determine the point of irreversible damage. To better understand what occurs within a leaf during exposure to thermal extremes, it would be valuable to characterise the T-F_0 curve and identify the CT_{MIN} and CT_{MAX} values for a plant. One could then ramp and hold leaf samples at temperatures at these thresholds for a set time, then measure F_V/F_M with the same Maxi-Imaging fluorescence system to examine potential recovery from exposure to damaging temperatures (e.g., Buchner et al., 2015). Then, one could investigate the correlation between *CT* and *LT* metrics and the reversibility of thermal damage. A more complete understanding of thermal tolerance responses and species specificity would be enhanced by exploring tissue biochemistry, the regulation of heat shock proteins, and gene expression at thermal extremes.

Towards standardised approaches for comparative thermal tolerance research

There will never be a perfect one-size-fits-all method for comparative measures of plant photosynthetic thermal tolerance, but our exploration of method variation we find there is a reasonable set of conditions that will fit most. We advocate that researchers use well-hydrated leaves (unless hydration status is an element of their experiment) and dry surface for these measures. Doing so allows easy comparison across experiments and gives a more indicative measure of the lowest potential CT_{MIN}.

We sought the maximum ramp rate that was repeatable and reliable. Our results suggest that there is a point beyond which temperatures are ramped too quickly and the T_{crit} value is exaggerated due to the change in F_0 lagging the change in leaf temperature, especially in heat tolerance assays. For an experiment on a single or few species, pilot studies on the effects of ramp rates are advisable. For broad interspecific studies, particularly in natural systems where other variables such as thermal history and the environment cannot be controlled, using a common temperature ramp rate is the only feasible approach. For such comparative work, we recommend ramping temperature at 30°C h^{-1} for CT_{MAX} and at or below 15°C h^{-1} for CT_{MIN}. We recognise that this is a slower ramp rate than often used for CT_{MAX} and a faster than usual CT_{MIN} measure. However, we found that the 15°C h^{-1} rate was not significantly different to slower rates for CT_{MIN} and thus represents the most efficient rate that could yield results reflective of natural scenarios. For CT_{MAX}, we argue that the 30°C h^{-1} rate enables physiological mechanisms that would normally provide thermal protection to the photosystem and cell membranes to be induced, without lag exaggerating CT_{MAX}, and may therefore be a more realistic or relevant

measurement of thermal tolerance than that provided by faster rates. These rates remain practical for achieving high throughput, especially with sample sizes that can be accommodated by large Peltier plates combined with the multi-sample imaging of Maxi-Imaging fluorimeters.

Clearly, any experimental thermal tolerance assay cannot perfectly mirror the conditions of a natural extreme thermal event. Rates of heating and cooling of plant tissues in nature are non-linear, not sustained, and strongly mediated by external conditions such as wind, solar radiation, season, and elevation (Sakai and Larcher, 1987; Leuning and Cremer, 1988; Vogel, 2009). The researcher must always remain appreciative of how extrinsic factors could affect these values and interpretations thereof for their study system. However, T-F_0 curves and derived T_{crit} values can indicate what the *potential* thermal limits of leaves are, under absolute conditions. The method provides power for comparative research, and also ample opportunity to explore the underlying mechanisms of species level differentiation. Moving toward a deeper understanding of the physiological processes conferring thermal tolerance is crucial in the changing climate where extreme weather events are increasing in frequency and intensity (Buckley and Huey, 2016; Harris et al., 2018).

Conclusions

The Peltier plate-Maxi-Imaging fluorimeter system described and tested here allows relatively high-throughput measurement of T-F_0 and the leaf critical thermal limits. This system offers great flexibility and substantially extends on previous versions. We have demonstrated that use of wet *vs* dry surface can significantly affect the CT_{MIN} estimate, but not CT_{MAX}, and that temperature ramp rate has strong species-specific effects on both CT_{MIN} and CT_{MAX}. Awareness of the physiological processes that underlie the rapid rise in F_0 and consideration of interspecific differences in leaf physiology and biochemistry are essential for making effective choices in the rate of heating or cooling leaf samples. We recommend use of parameters that maximise repeatability and efficiency of the measurements without introducing artefacts of ramp rate. As plants around the world are exposed to more thermal extremes by the effects of climate change, versatile ecophysiological tools such as this Peltier plate-Maxi-Imaging fluorimeter system will be valuable for generating new insights in plant responses and thermal tolerance.

Materials and Methods

Species description and leaf samples

Wahlenbergia ceracea Lothian (Campanulaceae) waxy bluebell is a small perennial herb that is sparsely distributed across south-eastern Australia. We grew F2 generation *W. ceracea* plants

under controlled glasshouse conditions (20/15°C set day/night temperatures) and leaves from mature plants were used for all experiments. Seed stock originated from Kosciuszko National Park, NSW, Australia (36.432°S, 148.338°E) that was collected in 2015 and 2016. *Melaleuca citrina* (Curtis) Dum. Cours. (Myrtaceae) common red bottlebrush were used for all experiments. This species is native to south-eastern Australia but also distributed as a cosmopolitan plant. Sampled individuals were growing as native shrubs at The Australian National University, ACT, Australia (35.279°S, 149.118°E). *Quercus phellos* L. (Fagaceae) willow oak trees were used only in the heat tolerance component of the surface wetness experiment, prior to the abscission of leaves in autumn. This deciduous species is native to North America and sampled individuals were growing as tall, shady ornamental trees at The Australian National University, ACT, Australia (35.277°S, 149.115°E). *Escallonia rubra* var. 'pink pixie' (Ruiz & Pav.) Pers. (Escalloniaceae) pink escallonia were used for the cold tolerance component of the surface wetness experiment and the ramp rate experiment in place of *Q. phellos* after the former shed its leaves. *Escallonia rubra* is native to South America and sampled individuals were growing as dense ornamental shrubs at The Australian National University, ACT, Australia (35.277°S, 149.117°E).

All measurements were taken between February and October 2019. Due to the variation in species availability across experiments and the potential effects of seasonal change on absolute tolerance values, we consider each experiment separately and do not draw comparisons across surface wetness and ramp rate experiments. Assays (surface wetness or ramp rates for heat or cold tolerance assays) were conducted on replicate days to control for potential effects of day. Leaves selected for measurement were fully expanded, visually free of damage and discolouration, and within two leaf pairs of a growing stem tip on an intact and healthy stem. Although leaf age could not be determined directly, these criteria allowed us to select leaves from the same cohort and of similar condition. Leaves were excised between 0900 and 1300 hours, placed in sealed bags, and then taken to the lab in an insulated container, where they were always used for T-F_0 measurements within 30 minutes of initial collection.

Temperature-dependent change in chlorophyll fluorescence (T-F_0) measurement

Leaf samples were attached to white filter paper (125 × 100 mm) with double-sided tape. We placed the filter paper with leaves on a Peltier plate (CP-121HT; TE-Technology, Inc., Michigan, USA; 152 × 152 mm surface) that was controlled by a bi-polar proportional-integral-derivative temperature controller (TC-36-25; TE-Technology, Inc.) and powered by a fixed-voltage power supply (PS-24-13; TE-Technology, Inc.). The Peltier plate uses four direct-

contact thermoelectric modules that can both cool and heat the plate, which with a MP-3193 thermistor (TE-Technology, Inc.) the plate had potential thermal limits of -20°C and 100°C. LabVIEW-based control software (National Instruments, Texas, USA) was adapted to control temperature ramp rate using source code available from TE-Technology, Inc. based on the supplied user interface. The Peltier plate maintained a stable set temperature within ± 0.1°C. We attached two type-T thermocouples to the underside of two randomly selected leaves on the plate as representative measures of leaf temperatures. Thermocouple temperature data were recorded every 10 s by a dual-channel data logger (EL-GFX-DTC; Lascar Electronics Ltd., Salisbury, UK) and the mean temperature of the thermocouples was used for leaf temperature calculations. Because the two thermocouples measured temperatures of two single leaves per experimental run, we were able to extract a small subset of ice nucleation temperatures (*NT*) using the temperature of the first exothermic reaction in cold tolerance assays. The Peltier plate assembly height was controlled by a laboratory scissor-jack to fit within an aluminium frame at an ideal height below the fluorescence camera (Fig. 1A). Heavy double-glazed glass was placed on top of the leaf samples on the plate to compress samples against the plate surface to ensure maximum contact and create a thermal buffer to ensure close matching of leaf and plate temperatures. In addition to greater thermal buffering relative to standard glass, double-glazed glass avoids condensation that might lead to erroneous measurements of F_0. All areas of both the Peltier plate and glass that were outside of the filter paper area were blacked out with heat-resistant black electrical tape to remove ambient light reflection and interference.

We used a Pulse Amplitude Modulated (PAM) chlorophyll fluorescence imaging system (Maxi-Imaging-PAM; Heinz Walz GmbH, Effeltrich, Germany) mounted 185 mm above the Peltier plate (imaging area of approximately 120 × 90 mm) to measure fluorescence parameters. A weak blue pulse modulated measuring light (0.5 μmol photons m^{-2} s^{-1}) was applied continuously at low frequency (1 Hz) to measure basal chlorophyll fluorescence (F_0) from the LHCII without driving PSII photochemistry. A red Perspex hood filtered ambient light from the samples and the camera, and the entire Maxi-Imaging-PAM assembly was covered by thick black fabric so that all measurements were made in darkness. Leaves were dark adapted for 30 minutes to oxidise all PSII acceptors and obtain the basal F_0 values and then a saturating pulse at 10,000 μmol photons m^{-2} s^{-1} was applied for 720 ms to determine the maximal fluorescence (F_M) when the photosystem reaction centres are closed. Variable fluorescence (F_V) was calculated as F_M - F_0 and the relative maximum quantum yield of PSII photochemistry (F_V/F_M) was derived. F_V/F_M is frequently used as a rapid measurement of stress or relative health of leaves, where optimal F_V/F_M values of non-stressed leaves are around 0.83 (Baker,

2008; Murchie and Lawson, 2013). Because our intention was to compare methods, we aimed for a uniform sample of leaves, and therefore we used F_V/F_M values > 0.65 to subset data to exclude any damaged leaves and focus on the T-F_0 of only healthy leaves. This conservative sample exclusion process resulted in some experimental conditions or species with uneven and lower sample sizes.

In each assay, we selected circular areas of interest that were as large as could fit within the boundaries of each leaf using the Maxi-Imaging-PAM software, such that the F_0 values were measured on the widest part of each leaf. Shortly after taking F_V/F_M, temperature ramps were started simultaneously with the continuous recording of F_0 values at set intervals with specifics varying depending on duration of the assay reflecting memory capacity limits of the Maxi-Imaging-PAM (see below). For hot T-F_0 measurements, the initial set temperature held for dark adaptation of the leaves and F_V/F_M was 20°C, which was then ramped to 60°C at varying rates (see ramp rate experiment). For cold T-F_0 measurements, the assays were conducted in a cold room (set temperature: 4 ± 2°C) so that the Peltier plate could reach -20°C. At ambient room temperatures of ~20-22°C, the Peltier plate can reach approximately -14°C before the plate heat output restrains cooling capacity. The initial set temperature held for dark adaptation of the leaves and F_V/F_M was 4°C, which was then ramped down to -20°C.

The T-F_0 curve produced by ramping the temperature of the Peltier plate (and leaf samples) is characterised by a stable or slow-rise in F_0 values until a critical temperature threshold where there is a fast rise in F_0. With temperature on the x-axis and F_0 on the y-axis, the inflection point of extrapolated regression lines for each of the slow and fast rise phases of the temperature-dependent chlorophyll fluorescence response is the critical temperature, T_{crit} (Knight and Ackerly, 2002; Neuner and Pramsohler, 2006). The term T_{crit} is ambiguous outside of this context when both hot and cold thermal tolerance assays are conducted within the same study. Hereafter, we refer to T_{crit} only as the temperature extrapolated at the inflection point, and elsewhere use accepted nomenclature used in thermal biology, CT_{MAX} and CT_{MIN}, as upper (heat) and lower (cold) thermal limits of leaf thermal tolerance (e.g., Sinclair et al., 2016; Janion-Scheepers et al., 2018). Figure 1 presents representative T-F_0 curves and the calculations of T_{crit} values for freezing leaves, where the fast rise phase occurs abruptly (Fig. 1B), and for heating leaves where the fast rise phase is relatively gradual (Fig. 1C). The inflection point was calculated using a break-point regression analysis of the mean leaf temperature estimated from two thermocouples attached to leaves on the plate and relative F_0 values using the *segmented* R package (Muggeo, 2017) using the R Environment for Statistical Computing (R Core Team,

2020). We provide example files and example R code for extracting T_{crit} values from T-F_0 curves at https://github.com/pieterarnold/Tcrit-extraction.

Surface wetness experiment: effect of wet *vs* dry surfaces for leaves on CT_{MIN} and CT_{MAX}

Most experiments that measure T-F_0 have measured leaf samples with all excess surface moisture removed, on a dry surface. However, maintaining water content of detached leaves by providing a wet surface where leaves were placed on top could be a viable way to facilitate water uptake and keep leaf samples hydrated. In our experiment, leaves were placed on a filter paper surface. For the wet surface treatment, leaves were placed as described above and then the filter paper was saturated with MilliQ water-soaked paper towels with excess water absorbed with dry paper towel thereafter. We compared T-F_0 curves and T_{crit} estimates for both heat and cold tolerance assays at a ramp rate of 60°C h^{-1} where leaves were placed on top of either wet or dry filter paper surfaces. A small subset of leaves on wet and dry surfaces were also measured for CT_{MIN} and NT at 15°C h^{-1} in addition to the 60°C h^{-1} experiment.

Ramp rate experiment: effect of temperature ramp rate on CT_{MIN} and CT_{MAX}

Studies on thermal tolerance limits vary substantially in their set temperature ramping rate (Table S1), ranging from 30 to > 600°C h^{-1} in studies on heat tolerance (CT_{MAX}) and from 1 to 10°C h^{-1} in studies on cold or freezing tolerance (CT_{MIN}). The difference in magnitude between heat and cold tolerance reflects differences in natural potential rates of heating and cooling, where leaves may rapidly increase in temperature (> 240°C h^{-1} for a short period (Vogel, 2009)) but cooling occurs far more slowly (rarely exceeding 5°C h^{-1} (Buchner and Neuner, 2009)). It stands to reason that the more than 10-fold difference in heating or cooling rates used among studies would affect the estimates and thus comparability of T_{crit}, but this effect is not well understood. We chose a wide range of temperature ramp rates for both hot and cold with the aim to determine how the T_{crit} estimate for CT_{MIN} and CT_{MAX} changes with ramp rate. We compared T-F_0 curves and T_{crit} estimates from different ramp rates for both heat (6, 15, 30, 45, 60, 120, 240°C h^{-1}) and cold (3, 6, 15, 30, 60, 240°C h^{-1}) tolerance assays where the filter paper was dry, and measurements were conducted in darkness. For 240, 60, and 30°C h^{-1} temperature ramps, F_0 was recorded at 10 s intervals, 20 s for 15 and 6°C h^{-1} ramps, and 30 s for 3°C h^{-1} ramps due to the 1000 record limit after which the Maxi-Imaging-PAM software stops recording.

Statistical analyses

The dataset was trimmed by removing leaves that had initial F_V/F_M values below 0.65, which might indicate an unhealthy leaf, hence sample sizes varied among species and experimental conditions. Data that matched conditions used in all experiments were used for multiple analyses (e.g., hot assay, ramp rate of 60°C h^{-1}, dry filter paper could be used for all). Linear regression models were implemented using the *stats* package in the R environment for statistical and graphical computing (v3.5.1) (R Core Team, 2020). Models were specified with CT_{MIN} or CT_{MAX} as the response variable and fixed categorical predictors of either wet/dry or ramp rate depending on the experiment. F_V/F_M was always included as a fixed covariate. We first fit models combining the three species for a given experiment, and then we fit species-specific models. Preliminary models were linear mixed effects regression models that included individual plant as a random factor, but in almost all cases, the term explained essentially zero variance, so we removed the random term in favour of a simpler linear model. Tables report model parameter estimates with statistical significance at $p < 0.05$ indicated in bold and with * symbols. Supplementary tables (Tables S2–S4) report full statistical model output. Figures show means with non-parametric bootstrapped 95% confidence intervals derived from the *Hmisc* R package (Harrell, 2019). Finally, predicted thermal tolerance estimates were modelled as a quadratic function of ramp rate treated as a continuous variable for visualisation purposes.

Supplemental data

The following supplemental materials are available.

Supplemental Table S1: Sample of temperature ramp rate variation from the literature.

Supplemental Table S2: Full statistical reporting for effects of wet *vs* dry surface for CT_{MIN} and CT_{MAX}.

Supplemental Table S3: Full statistical reporting for effects of wet *vs* dry surface combined with temperature ramp rate on CT_{MIN}.

Supplemental Table S4: Full statistical reporting for effects of temperature ramp rate for CT_{MIN}.

Supplemental Table S5: Full statistical reporting for effects of temperature ramp rate for CT_{MAX}.

Supplemental Figure S1: Various experimental applications of the Peltier plate and chlorophyll fluorescence Maxi-Imaging-PAM system

Supplemental Figure S2: Effects of wet *vs* dry surface combined with temperature ramp rate on CT_{MIN} and *NT*.

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Tables

Table 1. Summary of analyses of all species and species-specific effects of wet *vs* dry filter paper surface on CT_{MIN} and CT_{MAX}.

Response: CT_{MIN}	**All species**	***W. ceracea***	***M. citrina***	***E. rubra***
Fixed effects	**Estimate**	**Estimate**	**Estimate**	**Estimate**
Dry surface / *E. rubra* (intercept)	**-18.36***	Intercept: -5.71	Intercept: **-20.36***	Intercept: **-31.26****
Wet surface	**3.81*****	**3.92*****	**2.98****	**3.99*****
F_V/F_M	6.19	-9.89	4.72	23.54
M. citrina	**-3.50*****	--	--	--
W. ceracea	-0.42	--	--	--
R^2	0.464	0.288	0.374	0.527

Response: CT_{MAX}	**All species**	***W. ceracea***	***M. citrina***	***Q. phellos***
Fixed effects	**Estimate**	**Estimate**	**Estimate**	**Estimate**
Dry surface / *M. citrina* (intercept)	**32.76*****	Intercept: 6.90	Intercept: **36.31***	Intercept: **47.34*****
Wet surface	-0.55	-1.47	0.32	-0.63
F_V/F_M	18.20	**46.02***	13.16	2.32
Q. phellos	**2.01****	--	--	--
W. ceracea	**-4.47*****	--	--	--
R^2	0.593	0.213	0.028	0.041

*Bold indicates significance at $p < 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Intercepts marked as significant are different from zero. Full statistical reporting is provided in Table S2.*

Table 2: Summary of analyses of all species and species-specific effects of variable temperature ramp rate on CT_{MIN} and CT_{MAX}.

Response: CT_{MIN}	**All species**	***W. ceracea***	***M. citrina***	***E. rubra***
Fixed effects	**Estimate**	**Estimate**	**Estimate**	**Estimate**
Ramp rate = 3°C h^{-1} / *E. rubra* (Intercept)	**-11.38*** **	Intercept: **-40.89** **	Intercept: **-16.82*** **	Intercept: **-11.58** **
Ramp rate = 6°C h^{-1}	-0.33	0.62	**-1.81***	-0.15
Ramp rate = 15°C h^{-1}	-0.32	-0.12	-0.80	-0.10
Ramp rate = 30°C h^{-1}	0.75	**1.67** **	0.91	-0.74
Ramp rate = 60°C h^{-1}	**-1.34** **	0.75	**-3.51*** **	**-2.47*** **
Ramp rate = 240°C h^{-1}	-0.80	0.70	**-1.74***	**-1.90** **
F_V/F_M	-0.89	32.04	4.66	0.12
M. citrina	**-2.18*** **	--	--	--
W. ceracea	**-1.53** **	--	--	--
Marginal R^2	0.230	0.126	0.332	0.220

Response: CT_{MAX}	**All species**	***W. ceracea***	***M. citrina***	***E. rubra***
Fixed effects	**Estimate**	**Estimate**	**Estimate**	**Estimate**
Ramp rate = 60°C h^{-1} / *E. rubra* (Intercept)	**27.79*** **	Intercept: 14.87	Intercept: **27.79*** **	Intercept: **41.75***
Ramp rate = 6°C h^{-1}	-0.68	**1.60***	**-7.71*** **	1.38
Ramp rate = 15°C h^{-1}	**-2.43*** **	**-2.00** **	**-4.68*** **	-1.40
Ramp rate = 30°C h^{-1}	**-1.74** **	**-2.11*** **	**-2.10** **	**-1.31***
Ramp rate = 45°C h^{-1}	**-1.48** **	-0.72	-0.95	**-2.68*** **
Ramp rate = 120°C h^{-1}	1.00	**1.78** **	**2.24***	-0.45
Ramp rate = 240°C h^{-1}	**2.03*** **	**2.78*** **	**3.76*** **	-0.13
F_V/F_M	**23.79*** **	**38.48*** **	**21.98** **	5.15
M. citrina	**-1.30*** **	--	--	--
W. ceracea	**-1.24** **	--	--	--
Marginal R^2	0.429	0.619	0.863	0.319

*Bold indicates significance at $p < 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Intercepts marked as significant are different from zero. Full statistical reporting is provided in Tables S3 and S4.*

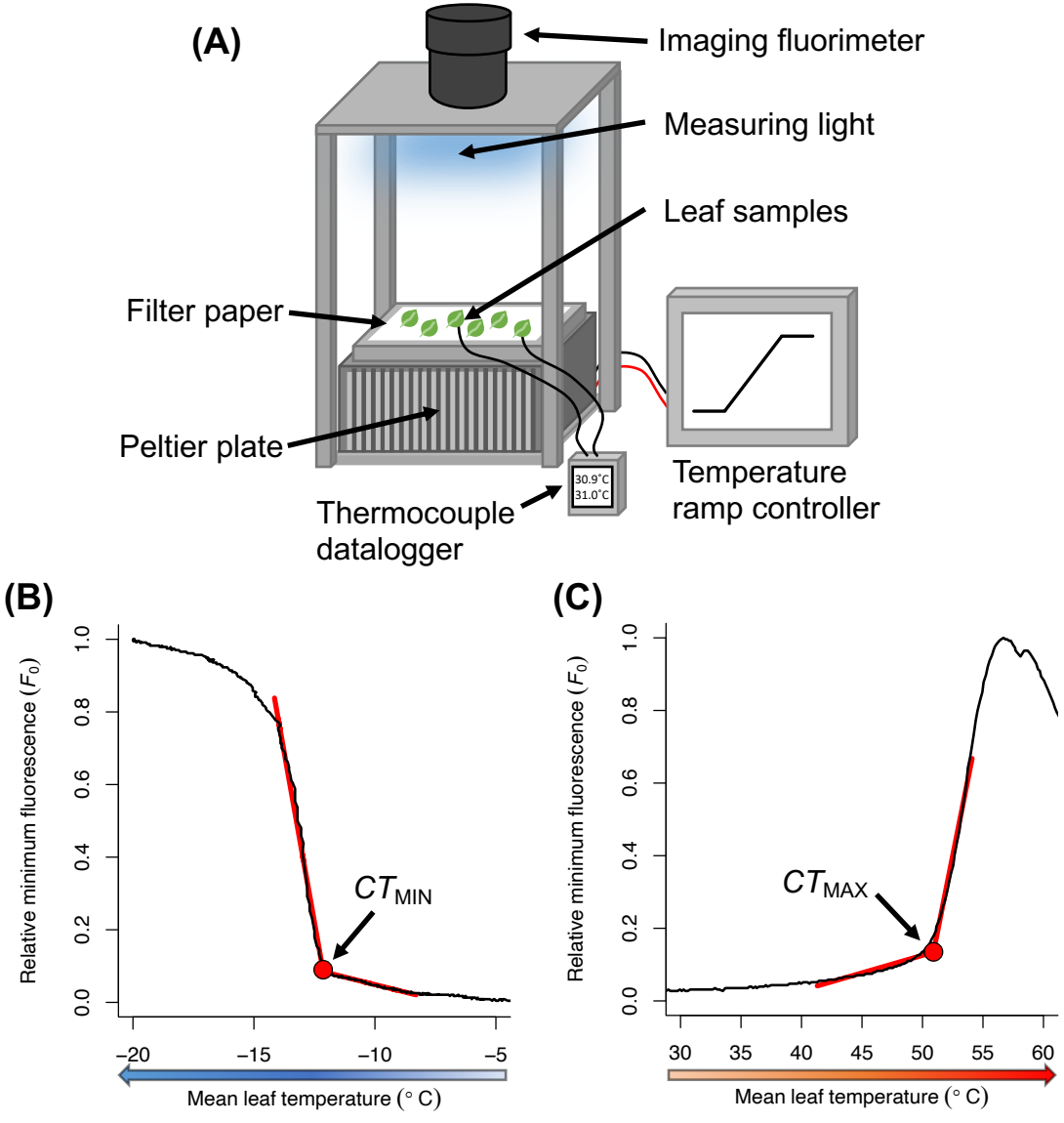
Figure legends

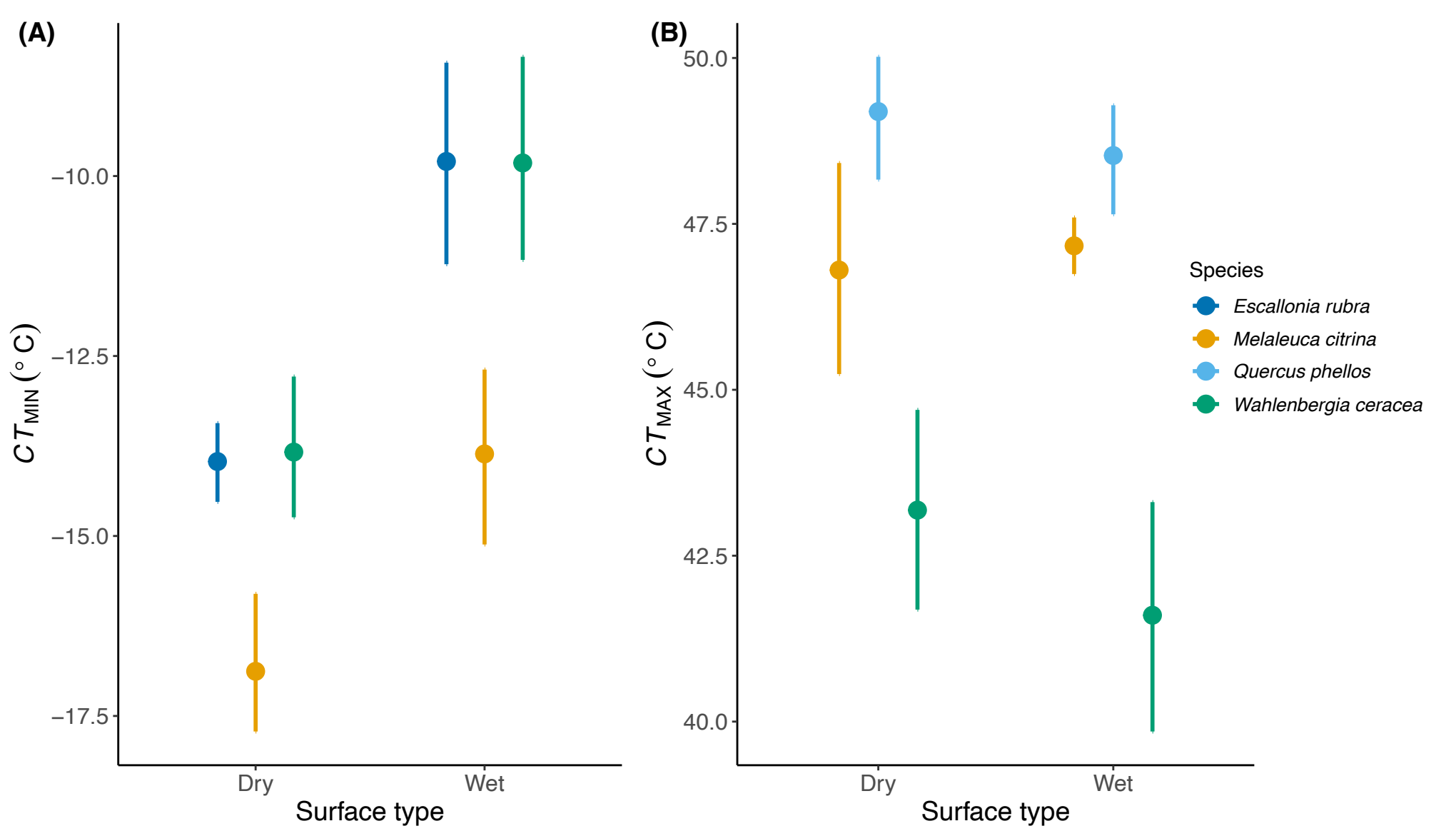
Fig. 1. Experimental system for measuring thermal tolerance limits and representative temperature-dependent chlorophyll fluorescence curves **(**T-F_0). (A) The Peltier plate-Maxi-Imaging fluorimeter setup for measuring leaf thermal tolerance limits. (B) Representative T-F_0 curve for CT_{MIN} (inflection point is the T_{crit}) where leaf sample temperature decreases to a point below freezing where the leaf rapidly emits more fluorescence, indicating the onset of freeze dehydration. (C) Representative T-F_0 curve for CT_{MAX} (inflection point is the T_{crit}) where leaf sample temperature increases beyond tolerance thresholds where the leaf rapidly emits more fluorescence, indicating the onset of heat necrosis. The direction of arrows below the *x*-axes indicate the direction of temperature change.

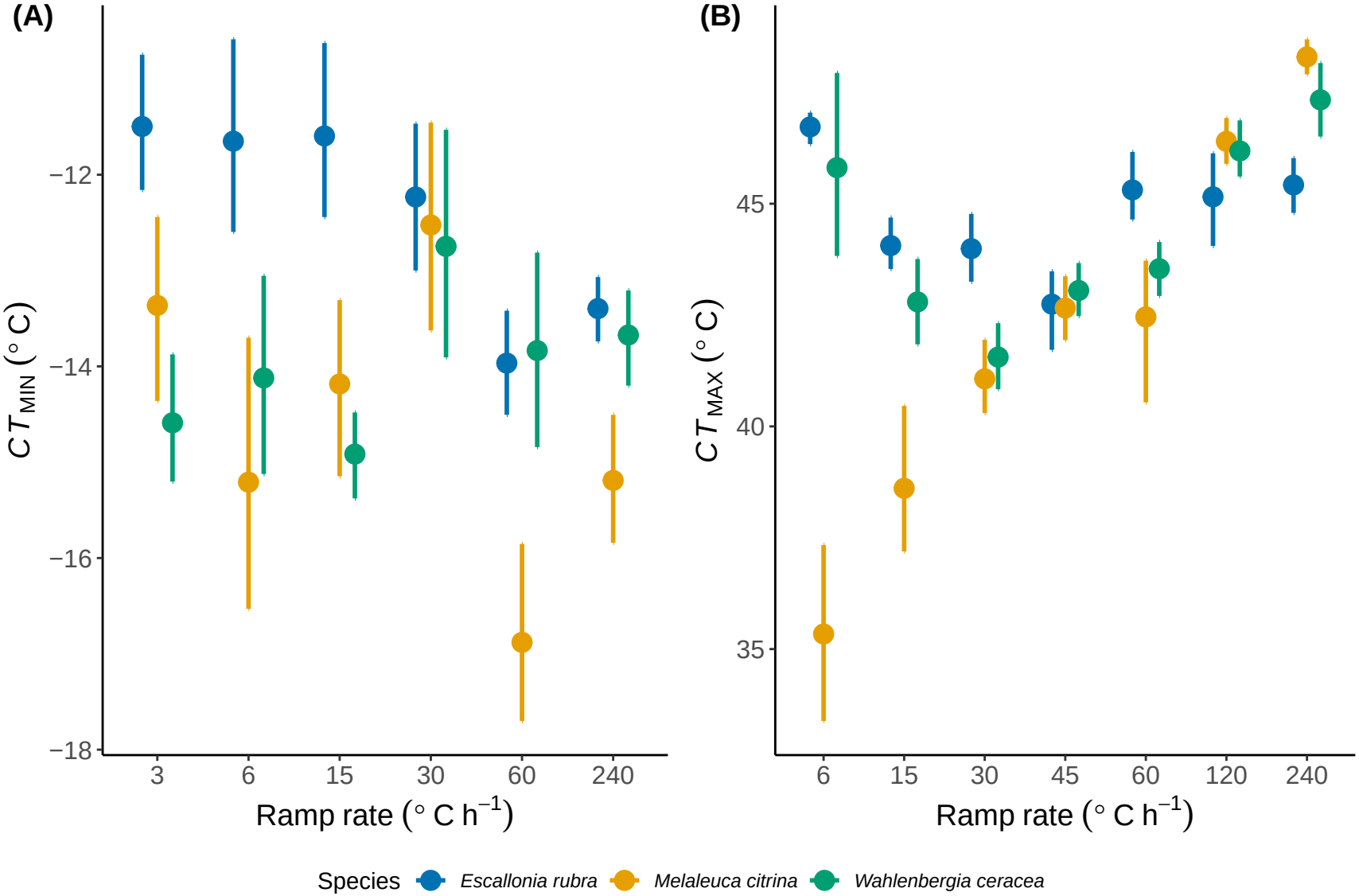
Fig. 2. The effect of varying surfaces (dry *vs* wet filter paper) on the CT_{MIN} and CT_{MAX} estimates (°C) from basal chlorophyll fluorescence (F_0) of leaves. We tested how (A) CT_{MIN} and (B) CT_{MAX} estimates of leaves from four plant species under standard dry conditions (dry filter paper surface) differed from wet conditions (wet filter paper surface). Data points are means and 95% CIs that overlay raw data (n = 12–25 per treatment × species combination).

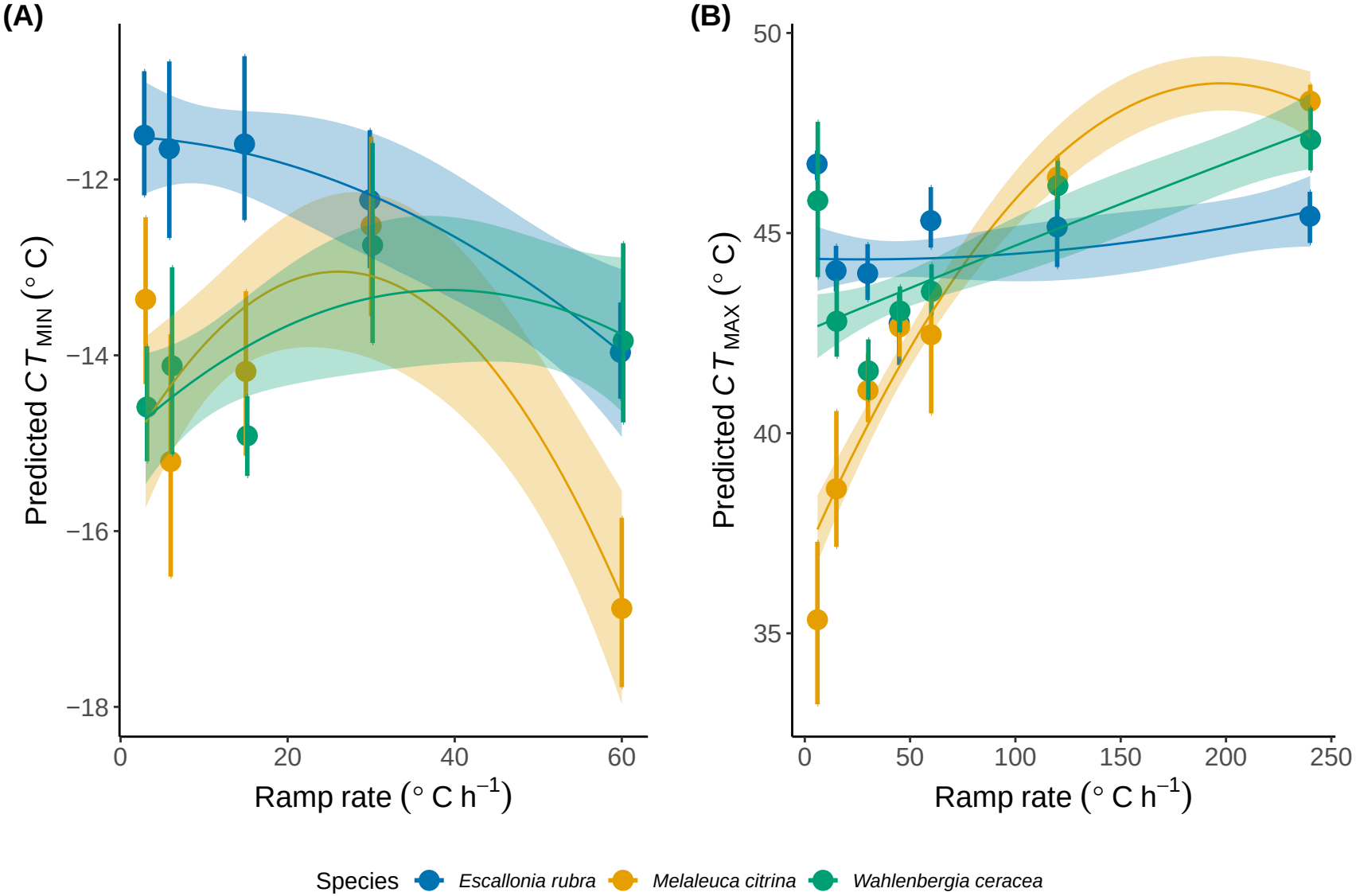
Fig. 3. The effect of varying temperature ramp rate (°C h^{-1}) on the CT_{MIN} and CT_{MAX} estimates (°C) from basal chlorophyll fluorescence (F_0) of leaves. We tested how (A) CT_{MIN} and (B) CT_{MAX} estimates of leaves from three plant species were affected by ramping the temperature stress at different ramp rates. Data points are means and 95% CIs that overlay raw data (n = 6–20 per treatment × species combination).

Fig. 4. The effect of temperature ramp rate (°C h^{-1}) as a continuous variable on the (A) predicted CT_{MIN} and (B) predicted CT_{MAX} estimates (°C) in leaves from three plant species. Data points are means and 95% CIs (n = 6–20 per treatment × species combination) with predicted response curves modelled with quadratic functions separately for each species.


(A)
Imaging fluorimeter
Measuring light
Leaf samples
Filter paper
Peltier plate
Thermocouple
datalogger
30.9°C
31.0°C
Temperature
ramp controller
(B)
Relative minimum fluorescence (F_0)
0.0
0.2
0.4
0.6
0.8
1.0
CT_{MIN}
−20
−15
−10
−5
Mean leaf temperature (° C)
(C)
Relative minimum fluorescence (F_0)
0.0
0.2
0.4
0.6
0.8
1.0
CT_{MAX}
30
35
40
45
50
55
60
Mean leaf temperature (° C)


(A)
−10.0
−12.5
−15.0
−17.5
CT_{MIN} (° C)
Dry
Wet
Surface type
(B)
50.0
47.5
45.0
42.5
40.0
CT_{MAX} (° C)
Dry
Wet
Surface type
Species
Escallonia rubra
Melaleuca citrina
Quercus phellos
Wahlenbergia ceracea


(A)
(B)
−12
−14
−16
−18
CT MIN (° C)
45
40
35
CT MAX (° C)
3
6
15
30
60
240
120
Ramp rate (° C h−1)
Species
Escallonia rubra
Melaleuca citrina
Wahlenbergia ceracea


(A)
(B)
Predicted CT_{MIN} (° C)
Predicted CT_{MAX} (° C)
−12
−14
−16
−18
50
45
40
35
0
20
40
60
0
50
100
150
200
250
Ramp rate (° C h^{-1})
Ramp rate (° C h^{-1})
Species
Escallonia rubra
Melaleuca citrina
Wahlenbergia ceracea

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