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Contribution of the alternative pathway to respiration during thermogenesis in flowers of the sacred lotus

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Abstract
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Contribution of the alternative pathway to respiration during thermogenesis in flowers of the Sacred lotus, *Nelumbo nucifera*.

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Abstract

We report the first in vivo measurements, using oxygen isotope discrimination techniques, of fluxes through the alternative and cytochrome respiratory pathways in thermogenic plant tissue; the floral receptacle of the sacred lotus, *Nelumbo nucifera*. Fluxes through both pathways were measured in thermoregulating flowers undergoing varying degrees of thermogenesis in response to ambient temperature. Significant increases in alternative pathway flux were found in lotus receptacles with temperatures 16-20 °C above ambient, but not in those with lesser amounts of heating. Alternative pathway flux in the hottest receptacles was 75% of the total respiratory flux. In contrast, fluxes through the cytochrome pathway did not change significantly during thermogenesis. These data support the hypothesis that increased flux through the alternative pathway is responsible for heating in the lotus, and that it is unlikely that uncoupling proteins, which would have produced increased fluxes through the cytochrome pathway, contribute to heating in this tissue. Alternative pathway capacity in lotus receptacles, determined from inhibitor titrations, was 72% of total respiratory flux in the heating tissues, which was similar to the alternative pathway capacity determined from the isotope discrimination data. In contrast, using inhibitors to determine alternative pathway capacity in non-heating tissues resulted in an overestimation of alternative pathway flux, confirming that while this technique is suitable for assessing alternative pathway capacity, it is not appropriate for quantifying fluxes through the two respiratory pathways in vivo.
Introduction

The first record of thermogenic activity in a plant was made by Jean-Baptiste Lamarck, in the European arum lily, probably *Arum italicum* (Lamarck de, 1815). Since then thermogenesis has been reported from species in the Annonaceae, Araceae, Arecales, Aristolochiaceae, Cycadaceae, Nymphaeaceae, Winteraceae, Illiciaceae, Magnoliaceae, Rafflesiaceae and Nelumbonaceae (Seymour, 2001a). Explanations advanced to explain the function of thermogenesis include: volatilization of scent compounds to attract pollinators (Meeuse and Raskin, 1988), prevention of low-temperature damage (Knutson, 1974) and provision of a thermal reward to insect pollinators (Seymour, 1997; Seymour et al., 2003). While thermogenic capacity can be large, heating tissues by up to 40 °C above ambient in some cases, flower temperature often varies considerably. In several species, however, respiratory heat-production is regulated to achieve fairly constant flower temperatures in widely variable environmental temperatures (Nagy et al., 1972; Knutson, 1974; Seymour and Schultzze-Motel 1996).

The strong correlation between heat generation and cyanide-resistant respiration in thermogenic plant tissues suggests that the alternative respiratory pathway is responsible for temperature increases in these plants (Nagy et al., 1972; Meeuse and Raskin, 1988). Cyanide-resistant respiration is mediated by a nuclear encoded, alternative oxidase (AOX) that exists as a homodimer in the inner mitochondrial membrane. The AOX is found in all plants and some protists, fungi and invertebrate animals (Vanlerberghe, 1997; McDonald and Vanlerberghe, 2004). During normal oxidative phosphorylation, electron transport is linked to the formation of a proton gradient that drives ATP production. In this instance, the terminal electron acceptor is the cytochrome c oxidase and about three ATPs are produced per O₂ consumed (Roberts and Wemmer, 1984). In contrast, electron transport to the AOX diverges from the phosphorylating pathway at ubiquinone and potential ATP production is reduced to about one per O₂ consumed and the extra energy is released as heat (Moore and Siedow, 1991). However, calorimetric studies of thermogenic flowers show that there is no net phosphorylation and all of the energy is released as heat (Seymour, et al., 1983; Lamprecht, et al., 1998).

Apart from its likely role in thermogenic tissues, the function of this apparently energetically wasteful pathway in plants was unclear until recently. However, an
increasing number of studies now support a role of the alternative pathway in preventing the build up of reactive oxygen species, by stabilizing the redox state of the mitochondrial ubiquinone pool whilst allowing continued operation of the citric acid cycle (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Skulachev, 1996; Maxwell et al., 1999; Parsons et al., 1999; Purvis, 2001; Ribas-Carbo et al., 2005). This role in stress mediation would explain the widespread occurrence of the alternative pathway in plants. Since the alternative pathway is able to maintain respiration free from the constraints imposed by low ADP and Pi availability, it has also been postulated that it allows high decarboxylation fluxes during C4 and Crassulacean acid metabolism (CAM) photosynthesis (Robinson et al., 1992; Chivasa et al., 1999) and respiration in plants growing under phosphorus limitation (Parsons et al., 1999; Juszczuk et al., 2001; Shane et al., 2004).

Regulation of AOX activity in isolated mitochondria can occur at a number of levels (for review see Millenaar et al., 2002; Lambers et al., 2005) however, the extent to which these operate in vivo is still unclear. Expression of the AOX has been shown to increase in plants exposed to low temperature, herbicides and inhibitors of the cytochrome pathway such as nitric oxide (Vanlerberghe and McIntosh, 1992; Aubert et al., 1997; Finnegan et al., 1997; Ribas-Carbo et al., 2000a; González-Meler et al., 2001; Huang et al., 2002; Zottini et al., 2002). However, changes in AOX protein levels do not necessarily reflect changes in activity in vivo (Millenaar et al., 2001; Guy and Vanlerberghe, 2005; Ribas-Carbo et al., 2005b). Further post-translational regulation is mediated by the redox state of the ubiquinone pool, which determines electron partitioning between the cytochrome and alternative oxidase pathways (Dry et al., 1989), and via effectors that have a direct impact on the activation state of the AOX protein. These include a regulatory disulfide bond that modulates the redox state of AOX (Umbach et al., 1994; Vanlerberghe et al., 1999) and α-keto acids such as pyruvate (Millar et al., 1993) that can further increase activity of the reduced form (Rhoads et al., 1998; Vanlerberghe et al., 1999).

There has been surprisingly little work with thermogenic plants, even though they stimulated much of the initial interest in the alternative pathway (Nagy et al., 1972; Meeuse and Raskin, 1988). In the voodoo lily, Sauromatum guttatum, high levels of AOX capacity (determined from inhibitor experiments) were found to coincide with spadix maturation (Rhoads and McIntosh, 1992). While in the dragon lily,
Dracunculus vulgaris, expression of the AOX gene occurred in highly thermogenic male florets, but not in non-thermogenic tissues of the same plant (Ito and Seymour 2005). Similarly, in this species, and other thermogenic arum lilies, the content of alternative oxidase protein increased with increasing thermogenic activity and declined immediately after heat production (Skubatz and Haider, 2001).

Recently, the role of AOX in thermogenesis has been brought into question by the discovery of uncoupling proteins (UCPs) in most plant tissues (Vercesi et al., 1995; Laloi et al., 1997), including thermogenic spadices of skunk cabbage Symplocarpus foetidus (Ito, 1999), and thermogenic receptacles of the sacred lotus, Nelumbo nucifera (Ito, pers comm.). Uncoupling proteins have long been known from animal tissues (e.g. brown adipose tissue) where they act as uncouplers of the mitochondrial electron transport chain, allowing energy derived from lipids to be used for heat generation instead of ATP production (Knutson, 1974; Klingenberg and Winkler, 1985). Furthermore, a number of studies have reported that the AOX and UCP co-occur in plant tissues (Sluse et al., 1998; Considine et al., 2001; Ito and Seymour 2005). UCP may short-circuit protons that could be transferred before the AOX, accounting for the complete conversion of energy entering the electron transport chain to heat with no ATP production (Seymour et al., 1983; Lamprecht et al., 1998).

Although UCP is equally expressed in both thermogenic and non-thermogenic tissues in the dead-horse arum, Helicodiceros muscivorus (Ito et al., 2003), it may have a thermogenic function in mitochondria with high AOX activity. However, the free fatty acids that stimulate UCP activity appear to inhibit the AOX, thus it is unlikely that both will reach maximal activity simultaneously (Kay and Palmer, 1985; Sluse et al., 1998). The involvement of AOX and UCP appears to depend on the substrate catabolised. UCP gene expression occurs strongly only during thermogenesis in the sterile male florets of Philodendron selloum, an arum lily that catabolises primarily lipid, while the AOX gene is expressed in Dracunculus vulgaris, another arum lily that uses carbohydrate (Ito and Seymour 2005).

It is now widely accepted that the use of chemical inhibitors is not an appropriate technique to measure flux through the respiratory pathways in vivo (Millar et al., 1995; Ribas-Carbo et al., 1995). Instead, stable oxygen isotope techniques have been developed (Guy et al., 1989) and improved (Robinson et al., 1992; Gonzalez-Meler et al., 1999; Henry et al., 1999; Ribas-Carbo et al., 2005a). The stable isotope method
relies on the fact that the two terminal acceptors of plant mitochondrial electron transport discriminate differentially against oxygen isotopes. Knowledge of the discrimination endpoints for cytochrome oxidase and AOX allows determination of the relative fluxes through the two pathways during steady-state respiration, in the absence of any chemical inhibitors. However, care must be taken to ensure that discrimination against oxygen is attributed purely to the respiratory chain, limiting the method to studies of dark respiration and to samples where diffusion of oxygen is not restricted.

These limitations in methodology have so far prevented use of the stable isotope method in studies examining the group of plants that arguably use the alternative respiratory pathway to the greatest extent, namely thermogenic plants. Often, the thermogenic organs in these plants are structurally dense, and diffusional limitations have obscured the isotopic signature (see review Robinson et al., 1995). However, in the sacred lotus, *Nelumbo nucifera*, strong thermoregulatory activity is found within the spongy tissues of the receptacle (Seymour and Schultze-Motel 1998) providing us with a model system in which to examine the role of the AOX and UCP in heat production.

In this study we used stable oxygen isotope techniques to determine electron partitioning between the cytochrome (and UCP) and alternative oxidases during thermoregulatory activity in the receptacles of the sacred lotus. We have also compared these measurements with traditional methods involving inhibitors.

**Results**

Respiration rates of whole lotus flowers, measured in the field, increased with tissue heating (Fig. 1). Laboratory measurements of oxygen consumption by receptacle sections that had been excised from flowers were similar to the rates observed in the field, within a range of thermogenic intensities (see controls in Fig. 2 and total respiratory flux in Fig. 3).

The capacities of the alternative and cytochrome pathways in lotus receptacles during the thermoregulatory phase were determined by titration with SHAM and KCN, respectively, on both strongly heating receptacles in the morning and weakly heating tissues in the afternoon. Total uninhibited respiration was 45% higher in the mornings than later in the day (Fig 2A). Residual respiration, in the presence of both 25 mM
SHAM and KCN concentrations at or above 4 mM, was 0.007 µmol O$_2$ g fw$^{-1}$ s$^{-1}$, which was 9-13% of the total, uninhibited rate (Figs 2A & B). Cytochrome pathway activity was removed by 4 mM KCN whilst 25 mM SHAM was needed to block alternative pathway activity (Figs. 2A & B). Alternative pathway capacity, measured in the presence of 16 mM KCN, was 72% of uninhibited total respiration in the morning but equivalent to total respiration in the afternoon (Fig 2B). Cytochrome pathway capacity, measured in the presence of 25 mM SHAM, was 39% and 56% of uninhibited total respiration in the morning and afternoon, respectively.

Oxygen isotope measurements were used to determine the actual fluxes through the alternative and cytochrome pathways in the absence of inhibitors. Total respiration rates determined by GCMS in the morning and afternoon were similar to those determined in respirometry measurements made in the laboratory and from whole flowers in the field (Fig 3). Lotus respiration rates measured by GCMS also showed a similar increase with thermogenesis to that observed in the field (Figs. 1 & 3). Total respiration increased significantly in tissues that were heating to temperatures of 16 °C or more above ambient in the morning, with alternative pathway flux exhibiting a similar significant increase with thermogenesis (Table 1). In the least active (afternoon) tissues, alternative pathway flux accounted for 43% of total respiration, but increased to 55-75% of the total in heating tissues. However, changes in cytochrome flux across the range of thermogenic activity were not significant.

**Discussion**

Our results demonstrate, conclusively, that there is increased flux through the alternative pathway in relation to heating of thermogenic tissues of lotus, and that the alternative pathway is responsible for the bulk of respiratory activity in these tissues. The use of oxygen isotope techniques in this study allowed us to confirm that previously reported high alternative pathway capacities in thermogenic tissues, measured using respiratory inhibitors, are matched by high in vivo alternative pathway fluxes. However, whereas the oxygen isotope technique allows accurate, and dynamic, determination of respiratory fluxes through both the alternative and cytochrome pathways, inhibitor studies can only be used to determine the maximum possible flux through each pathway (Millar et al., 1995; Ribas-Carbo et al., 1995). Until our work, oxygen isotope techniques had not been used to determine in vivo flux
through the alternative pathway in thermogenic plants due to the high diffusional resistances found in most thermogenic tissues (Guy et al., 1989). This limited the use of isotopic analysis to mitochondrial extracts from such tissues, and the only reported study of this kind was for mitochondria extracted from *Symplocarpus foetidus* spadix, in which 78% of the respiratory flux was attributed to the alternative pathway (Guy et al., 1989). In contrast, the thermogenic receptacle tissue of lotus flowers contains many air-spaces, making this plant an ideal model for the study of thermogenesis in vivo. The *in vivo* fluxes measured in our study showed that the alternative pathway contributed 55-75% of respiratory flux during heating, similar to the values found for *S. foetidus* mitochondria. Our inhibitor studies also suggested that 72% of the total respiratory flux in heating tissues could be attributed to the alternative pathway. This is in good agreement with the actual flux measured using oxygen isotopes. In contrast, alternative pathway capacity determined from the inhibitor studies, overestimated the contribution of this pathway in non-heating tissues due to electrons being diverted from the cytochrome to the alternative pathway (Fig 2).

Alternative pathway fluxes reported for tissues from non-thermogenic plants range from 0 to 50% of total respiration (Robinson et al., 1995), similar to the 43% flux we observed in non-heating receptacles (Table 1). The only report of higher alternative pathway fluxes in a non-thermogenic plant (63%), was for leaves of the CAM plant *Kalanchoe daigremontiana* (Robinson et al., 1992). Alternative pathway fluxes higher than those reported for thermogenic plants, including those found in our study, have only been observed in mutants of *Chlamydomonas reinhardtii* lacking cytochrome oxidase (Guy et al., 1992).

Our oxygen discrimination end-points for the alternative and cytochrome pathways, were similar to those previously reported for non-green tissues in a range of plants (Robinson et al., 1995). Higher end-points for the alternative pathway have been reported in green tissues; with the end-point increasing from 27‰ to 32‰ during greening of soybean cotyledons (Ribas-Carbo et al., 2000b). Following pollination, the lotus receptacle greens, offering us another system in which to study the impact of greening on the isotopic fractionation by the alternative pathway. However, none of the data collected in the current study were from green receptacles.

While the alternative pathway has traditionally been invoked as the most likely source of heat in thermogenic plants, the recent discovery of plant UCPs could provide an
alternative mechanism for heat generation (Vercesi et al., 1995; Laloi et al., 1997). If UCPs were responsible for heat generation in sacred lotus, then we would expect an increase in flux through the cytochrome pathway during thermogenesis. However, our data show no change in flux through the cytochrome pathway during heating, and thus provide no evidence for involvement of UCP in thermogenesis in lotus. This is consistent with the observation that the respiratory substrate is carbohydrate in the lotus (Seymour and Schultze-Motel, 1998) and that AOX expression is enhanced in thermogenic flowers that use this substrate, while UCP expression occurs during thermogenesis when lipid is the main substrate (Ito and Seymour 2005).

**Conclusion**

The use of oxygen isotope fractionation has allowed us to accurately determine the flux of electrons through the cytochrome and alternative pathways in the thermogenic floral receptacle of the sacred lotus, *Nelumbo nucifera*, confirming that the bulk of the heat production occurs through the alternative pathway. Flux through the cytochrome pathway did not change significantly during thermogenesis making a significant role for UCPs in heat production unlikely in these tissues. Our results also confirm that whilst inhibitor titrations are suitable for assessing alternative pathway capacity, they often overestimate the actual *in vivo* respiratory fluxes. The suitability of the thermogenic tissues of lotus for oxygen isotope fractionation measurements makes this plant an important and fascinating model for investigating the basis for cellular thermoregulation in plants.

**Materials and methods**

**Plant material**

Sacred lotus (*Nelumbo nucifera*, Gaertn.) flowers were obtained from a pond in the Adelaide Botanic Gardens, South Australia. Measurements were performed during the summer flowering periods (December-February) between 2003 and 2005.

At the time of sampling, the majority of flowers were in the thermoregulatory pistillate stage of development, characterised by slightly opened or bowl-shaped petals (stage 2 according to Seymour and Schultze-Motel, 1998). A small number of non-heating flowers (stages 1 and 3) were also monitored for comparison. At each
sampling time, the temperatures of the ambient shaded air, the experimental receptacle, and an adjacent, pre-thermogenic (stage 1) receptacle were measured with a needle thermocouple and a Fluke model 52 digital thermometer. Flowers for laboratory respiration and mass spectrometry measurements were taken immediately to the nearby laboratory at the University of Adelaide. In all cases, the period between cutting a flower and the end of measurements was less than 1.5 h.

*Field Respirometry*

Measurements of CO$_2$ release were made on whole, attached flowers of *N. nucifera* using a flow-through system described earlier (Seymour and Schultze-Motel 1998). Briefly, a pump drew atmospheric air through a plastic hood covering each flower and sent it through a mass flow meter that was vented to the atmosphere. Sub-samples of the flows through three hoods and one reference channel of atmospheric air were drawn into a CO$_2$ analyser (Anarad model AR50 IRGA). After respiration measurements were completed, receptacles were carefully dissected from each flower and weighed. Mass specific respiration rates were calculated on the basis of receptacle mass (g fwt), and a proportional contribution of receptacles to total floral respiration of 54% (Seymour and Schultze-Motel 1998).

*Titration with respiratory inhibitors*

Laboratory respirometry was performed within a 5 mL glass syringe equipped with a 3 mm (OD) oxygen electrode (model MI-730; microelectrodes.com), sealed with a Silastic sleeve in a hole at the end of the barrel, and a 3-way stopcock with a needle. Four syringes were suspended horizontally in a water bath with their plungers protruding through one side and their needle tips through the other. The water bath was connected to a thermocirculator (Julabo.com) and the electrode, syringe barrel and stopcock were thermostatted to 32 °C. The oxygen electrodes were connected to a Sable Systems ReadOx-4H meter (sablesys.com) and then to a PowerLab SP4644 interface (ADInstruments.com), for simultaneous recording from all four. They were calibrated with humidified, high-purity nitrogen at the beginning of a series of measurements from each flower, and with atmospheric air before each measurement.
A razor blade was used to cut vertical, pie-shaped wedges of tissue from receptacles. The slices, which contained spongy receptacle tissue and parts of ovules, were weighed. Receptacle tissue was then vacuum infiltrated in 5 mL glass syringes with either buffer (10 mM N-Tris-hydroxymethyl-2-aminoethanesulfonic acid (TES), 0.2 mM CaCl, pH 7.2) or selected concentrations of inhibitor (KCN, or salicylhydroxamic acid (SHAM)). After vacuum infiltration in the syringe (twice), the tissue sample was removed, placed in the fold of a Whatman No. 1 filter paper wrapped with cotton gauze and placed inside a plastic bag. Some of the solution was removed from the tissue by shaking the sample several times, and more was removed by capillarity in the filter paper. This procedure exposed all spongy tissue surfaces to the test solution, but ultimately refilled the larger pore spaces with air to eliminate liquid boundary layers during respirometry. There was no significant difference in respiration rates between non-infiltrated tissue and tissue that had been vacuum infiltrated with buffer. Respiration rates of blotted tissues were then measured in the respirometry syringes.

Recordings were taken over approximately 15 min. The average slope of the decrease in PO$_2$ was measured after 1.5 min of equilibration for approximately 10 min. Minimum acceptable PO$_2$ was 10 kPa to avoid diffusion limitation as much as possible. The slope tended to decrease during a run in the most active controls, but the changes were less than 20%. Oxygen consumption rate was calculated from the PO$_2$ slope and the gas volume of the syringe, accounting for tissue volume.

*Respiration and discrimination analysis*

Discrimination during respiration was determined in freshly harvested lotus receptacles during periods of high (early morning) or low (afternoon) thermogenesis (Seymour et al., 1998). The steady state flux of electrons through the cytochrome and alternative pathways in lotus receptacles was determined using the oxygen isotope technique established by Guy et al. (1989) and subsequently developed to measure the gas phase on-line (Robinson et al., 1992). A full review of the theoretical and practical aspects of this technology can be found in Ribas-Carbo et al. (2005a).

Respiration rates and differential uptake of oxygen stable isotopes were measured simultaneously in six sequential samples taken from the gas phase surrounding the respiring receptacle tissue. Small sections (approximately 1.5 cm$^3$) of freshly
harvested lotus receptacle tissue were weighed and placed inside a 25 ml gas-tight syringe. Air samples (100 µL) were withdrawn from the syringe at approximately 10 min intervals and injected into a GCMS system (NA 1500 Carlo-Erba Instrumentazione, Italy, Optima, Micromass, UK). The fraction of O$_2$ remaining (f) and its isotopic composition (R) were measured, and the isotopic discrimination factors and partitioning of electrons between the cytochrome and alternative pathways were calculated as previously described (Guy et al., 1989; Henry et al., 1999; Ribas-Carbo et al., 2005a). The r$^2$ of all unconstrained linear regressions between –ln f and ln (R/R$_0$), with a minimum of six data points, was at least 0.991.

To establish the discrimination endpoints for the alternative ($\Delta_a$) and cytochrome ($\Delta_c$) oxidases, receptacle tissue was vacuum infiltrated with either 16 mM KCN or 25 mM SHAM (made from a 1M stock solution in DMSO), respectively, prior to measurement. The endpoints obtained ($\Delta_c = 16.97 \pm 2.00‰$, and $\Delta_a = 26.49\pm1.25‰$) were then used to calculate the flux through the alternative and cytochrome pathways in uninhibited tissues as described in Ribas-Carbo et al. (2005a,b). The reproducibility of measurements of O$_2$ concentration and fractionation were determined using air samples withdrawn from the empty syringe and were ±2% and ±0.01%, respectively.

Statistical analysis

Changes in flux through the respiratory pathways were investigated by analysis of variance using JMP 5.1 (SAS Institute Inc.). Respiratory flux as a function of temperature was plotted and regressions fitted and tested using SigmaPlot 9.0 (SPSS).

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Figure legends

Figure 1. The relationship between receptacle respiration rate (µmol O₂ gfw⁻¹ s⁻¹) and the difference between lotus receptacle temperature (t_r, °C) and temperature of a near-by non-thermogenic receptacle (t_n, °C), measured in the field. (r²=0.96; y=0.0023+0.0252e⁰.⁰⁷⁶⁵⁵x).

Figure 2. The effect of A) KCN on cytochrome pathway respiration measured in the presence of 25 mM SHAM and B) SHAM on cyanide-resistant pathway respiration measured in the presence of 16 mM KCN, in lotus receptacle tissue. Total respiration rates of uninhibited receptacle tissues collected in the morning (filled triangle, a.m.) and in the afternoon (open triangle, p.m.) are also shown. For inhibitor titrations, tissue sections were vacuum-infiltrated with inhibitor, blotted dry and then O₂ uptake rate measured in air at 32°C, in a closed respirometer. Respiration rate was calculated over the range 20-14 kPa O₂ partial pressure (~15 min). Data points are means ± SE, n=6.

Figure 3. Total respiratory flux (µmol O₂ gfw⁻¹ s⁻¹) and fluxes through the alternative (AOX) and cytochrome (COX) pathways, in lotus receptacle tissues as a function of the difference between receptacle temperature (t_r, °C) and temperature of an adjacent non-heating receptacle (t_n, °C). Total respiration (solid line, r²=0.95; y=0.0253+0.0004e⁰.⁰⁶⁴⁵x), alternative pathway flux (dashed line, r²=0.86; y=0.0161+0.0003e⁰.⁰⁶⁵x), and cytochrome pathway flux (dotted line, r²=0.75; y=0.0092+0.0001e⁰.⁰⁶¹³x). Partitioning between the two pathways was determined on the basis of Δ¹⁸O measurements of intact tissues.
Table 1. Mean total respiration rates and fluxes through the alternative and cytochrome pathways (µmol O₂ gfw⁻¹ s⁻¹) with different amounts of tissue heating in lotus receptacles. Temperature categories represent the difference between receptacle temperature (tᵣ) and the temperature of an adjacent non-thermogenic receptacle (tₙ). Values are means ± (SE), n=2-5. NS=not significant.

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ᵃIndicates significant difference within row (Tukey test, P< 0.05).
Figure 1.
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Figure 3.