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Distribution of thermogenic activity in floral tissues of Nelumbo nucifera

Nicole M. Grant
University of Wollongong, nmg944@uow.edu.au

Rebecca E. Miller
Wollongong, bec@uow.edu.au

Jennifer Watling
University of Adelaide

Sharon A. Robinson
University of Wollongong, sharonr@uow.edu.au

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Abstract

Thermogenesis in Nelumbo nucifera (Gaertn.) has been known to scientists for many years; however, the extent of heating by different floral parts remains unclear. We present evidence that the receptacle, stamens and petals produce heat independently, and that the source of heating in these tissues is most likely the alternative oxidase (AOX). The temperatures of the receptacle, petals and stamens were significantly higher than non-thermogenic leaf tissue. After removal from the pedicel, the receptacle retained the most heat (8.1 ± 1.9°C above non-thermogenic tissue temperature) and the petals the least (2.8 ± 4.2°C), with the stamens intermediate. High AOX protein levels and flux through the AOX pathway (in all tissues) during the thermogenic period are consistent with AOX being the mechanism used for thermogenesis. Lipids and carbohydrates were investigated as possible substrates for thermogenesis. There was little change in total lipids during floral development; however, soluble carbohydrate levels decreased by 70% with the onset of thermogenesis. These sugars may fuel thermogenesis in the stamens. The localisation of AOX protein in the various floral parts and the evolutionary significance of its heating role are discussed.

Keywords

alternative oxidase, alternative pathway respiration, lipid, plant thermogenesis, sacred lotus, starch

Disciplines

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Chapter 4. Distribution of thermogenic activity in floral tissues of *Nelumbo nucifera*

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ABSTRACT

Thermogenesis in *Nelumbo nucifera* has been known to scientists for many years, however the extent of heating by different floral parts remains unclear. We present evidence that the receptacle, stamens and petals produce heat independently and that the source of heating in these tissues is most likely the alternative oxidase (AOX). The temperatures of the receptacle, petals and stamens were significantly higher than non-thermogenic leaf tissue. After removal from the pedicel, the receptacle retained the most heat (8.1 ± 1.9°C above non- thermogenic tissue temperature) and the petals the least (2.8 ± 4.2°C), with the stamens intermediate. High AOX protein levels and flux through the AOX pathway (in all tissues) during the thermogenic period are consistent with AOX being the mechanism used for thermogenesis. Lipids and carbohydrates were investigated as possible substrates for thermogenesis. There was little change in total lipids during floral development, however soluble carbohydrate levels decreased by 60% with the onset of thermogenesis. These sugars, may fuel thermogenesis in the stamens. The localisation of AOX protein in the various floral parts and possible evolutionary significance are discussed.

INTRODUCTION

Thermogenesis, or self-heating, in plants has been known to scientists for more than 200 years. Heating occurs across a diverse group of plants with examples found amongst water plants (Nelumbonaceae; Miyake, 1898), cycads (Tang, 1987), parasitic plants (Patino *et al.*, 2000), magnolias (Dieringer *et al.*, 1999), and aroids (Araceae), the latter accounting for the largest proportion of thermogenic species. Heating generally occurs in reproductive structures such as the fertile male florets in *Philodendron bipinnatifidum* (*syn. P. selloum*; Chapter 5) and *P. melinonii* (Seymour and Gibernau, 2008) and the ovule-containing receptacle in *Nelumbo nucifera* (Seymour and Schultze-Motel, 1996; Grant *et al.*, 2008; Chapter 2; Grant *et al.*, 2009; Chapter 3; Appendix 1). However, heating in non-reproductive parts has also been reported including the appendix of the spadix in *Dracunculus vulgaris* (Seymour and Schultze-Motel, 1999) and sterile male florets of *P. bipinnatifidum* (Nagy *et al.*, 1972). Heating may have evolved to assist in attraction of insect pollinators either by the volatilisation of scent compounds (Meeuse and Raskin, 1988) or as a heat reward (Ervik and Barfod, 1999). For species such as the skunk cabbage (*Symplocarpus foetidus* and *S. renifolius*) which flowers in early spring, heating is thought to prevent low temperature damage during pollen maturation (Knutson, 1974; Onda *et al.*, 2008; Seymour *et al.*, 2009a). In addition to heating, some thermogenic plants can also regulate the temperature of their floral parts, maintaining a constant temperature despite changing ambient temperatures. This thermoregulation has been reported in *P. bipinnatifidum* (Nagy *et al.*, 1972), *S. foetidus* (Knutson, 1974) and *N. nucifera* (Seymour and Schultze-Motel, 1996). This temperature regulation must occur at the cellular level, as plants do not contain the complex regulatory mechanisms of endothermic animals.

There are two known means by which plants can produce heat; the alternative oxidase and uncoupling proteins (Watling *et al.*, 2006; Onda *et al.*, 2008). Both mechanisms utilise respiratory energy by uncoupling electron transport from ATP production. The alternative oxidase (AOX) is a cyanide resistant terminal oxidase located on the matrix side of the inner mitochondrial membrane. The AOX protein accepts electrons from ubiquinol and reduces oxygen to water, however AOX is non-proton motive and potential energy from electron transport is released as heat (Moore and Siedow, 1991). The AOX protein is present in all plants and *AOX* genes have recently been discovered

in all kingdoms except the Archaebacteria (McDonald and Vanlerberghe, 2006; McDonald, 2008). Plant uncoupling proteins (pUCPs) belong to the mitochondrial anion carrier family of proteins and are also located in the inner mitochondrial membrane (Vercesi *et al.*, 1995). They partially uncouple respiration from ATP synthesis by dissipating the electrochemical H^+ gradient (Jezek *et al.*, 1996). AOX and pUCP are widespread in plant tissues of both thermogenic and non-thermogenic species.

Which pathway is responsible for heat generation seems to vary with species. For example, in *P. bipinnatifidum* (Chapter 5) and *S. renifolius* (Ito-Inaba *et al.*, 2008a; Onda *et al.*, 2008) both AOX and pUCP proteins were expressed in thermogenic tissue and thus might operate together to produce heat. In *S. renifolius,* substantial linoleic acid (LA) inducible uncoupling is thought to be primarily mediated by pUCPs (Onda *et al.*, 2008). In addition, although LA is generally assumed to inhibit AOX activity (Sluse *et al.*, 1998), pyruvate activated AOX activity was only partially inhibited by LA in *S. renifolius* (Onda *et al.*, 2008), indicating that activities of both AOX and pUCP can remain high simultaneously. However, presence of the protein does not directly correspond to heating by AOX or pUCPs, and it is necessary to measure activity *in vivo* to demonstrate such a role. Stable isotope methodology, which makes use of differential fractionation of O isotopes by the terminal oxidases of the electron transport chain (AOX and COX), allows *in vivo* measurements of the involvement of the alternative pathway (Robinson *et al.*, 1992; Ribas-Carbo *et al.*, 1995; Robinson *et al.*, 1995). In *N. nucifera* receptacles, AOX protein levels were high during the thermogenic period, whilst pUCPs were not detected and AOX flux increased with heat production whilst COX pathway flux remained stable (Watling *et al.*, 2006; Grant *et al.*, 2008; Chapter 2). The alternative oxidase thus appears to be solely responsible for uncoupled respiration and heating in *N. nucifera* receptacles. Measurements of AOX flux in *P. bipinnatifidum* similarly suggest that the bulk of the flux is through the AOX despite the presence of pUCP protein in these tissues (see Chapter 5). In *S. renifolius*, both AOX and pUCP are thought to play a role in thermogenesis (Onda *et al.*, 2008) as AOX in this species does not appear to be as sensitive to LA as in non-thermogenic species (Sluse *et al.*, 1998), however which pathway(s) are operational *in vivo* has not been tested.

Thermogenic plants can use carbohydrate and/or lipids as respiratory substrates for thermogenesis. For example, in thermogenic clubs of *Arum maculatum* (ap Rees *et al.*,

1977), and receptacles of *N. nucifera* (Grant *et al.*, 2008; Chapter 2), both of which use AOX to generate heat, starch content declines significantly by the end of the thermogenic period. Based on respiratory quotients, *S. foetidus* also uses carbohydrates (Seymour and Blaylock, 1999). In contrast, the spadix of *P. bipinnatifidum* switches from carbohydrate to lipid metabolism with the start of thermogenesis (Walker *et al.*, 1983; Seymour *et al.*, 1984), and lipid content decreases significantly by the end of the thermogenic period (Chapter 5). There is some suggestion in the literature that substrate type determines whether AOX or pUCPs are used for thermogenesis, as both can be present in thermogenic tissues. For example, *AOX* , but not *pUCP*, gene expression increased in thermogenic tissue of *D. vulgaris* which uses carbohydrates for heating (Ito and Seymour, 2005). In contrast, *pUCP*, but not *AOX*, gene expression increased in *P. bipinnatifidum* (Ito and Seymour, 2005), where lipids are the respiratory substrate (Walker *et al.*, 1983). However, measurements of protein content in thermogenic tissues of *P. bipinnatifidum* suggest that AOX protein increases rather than pUCP (Chapter 5), indicating that substrate alone may not determine whether AOX or pUCPs are used.

Heating generally occurs in floral tissues and until recently, it was thought the flowers of thermogenic plants possessed some unifying characteristics (Seymour and Schultze-Motel, 1997). Flowers of thermogenic species are usually large as small flowers with high surface area:volume ratios are unable to retain heat efficiently (Seymour and Schultze-Motel, 1997). Thermogenic flowers are also usually protogynous; the female parts of the flower are receptive before the male parts. However, the discovery of thermogenesis in the stalk of the parasitic plant *Rizanthes lowii* (Rafflesiaceae; Patino *et al.*, 2000) shows that thermogenesis may occur in other plant organs.

The pattern and location of heating is varied and species specific (see Table 1.1 page 2). Heating episodes often correspond with reproductive maturity of the stigma and pollen and occur in these specific floral parts at different times. *Dracunculus vulgaris* displays a triphasic heating pattern, the floral chamber heating on the first night, appendix heating the following day and a second floral chamber heating episode on the second night (Seymour and Schultze-Motel, 1999). In other species heating is less stage specific, as in *S. foetidus* where thermogenesis can occur in all stages of flowering (Seymour and Blaylock, 1999). In addition, the extent of heating can be tissue and floral stage specific. For example, female stage flowers of *Magnolia tamaulipana* heat almost

twice as much as male stage flowers (1.0-9.3 °C and 0.2-5.0 °C above ambient respectively; Dieringer *et al.*, 1999) whilst male florets of *P. bipinnatifidum* can reach more than 15°C above ambient compared with the female florets which do not heat at all (Chapter 5). Heating in these plants clearly has a specific purpose and the location and duration of the heating episodes reflects this. When heating is a protecting mechanism against low temperatures, it is less stage/tissue specific and of long duration, as in *S. foetidus*. However, if heating aids in the volatilisation of scent compounds to attract insect pollinators, it needs to be executed at specific times of day as is shown with the appendix heating episode in *D. vulgaris*.

Heating in thermogenic plants is often reported on a whole flower basis so the specific localisation of the thermogenic tissue remains unclear. In *S. renifolius* and *S. foetidus* the thermogenic spadix is small (2.5 -4.0 g) with floret mass even lower (Seymour and Blaylock, 1999; Onda *et al.*, 2008). This small size makes distinguishing between adjacent thermogenic and non-thermogenic tissues difficult. As both pUCP and AOX have been found in the petals and pistil of *S. renifolius*, it has been suggested that heating occurs in both tissues (Onda *et al.*, 2008). In *P. bipinnatifidum* the sterile male florets have been the focus of most studies (Nagy *et al.*, 1972; Seymour, 1999), however recently precise thermoregulation has also been reported in the fertile male florets of this genus (Seymour and Gibernau, 2008; Chapter 5).

The sacred lotus (*Nelumbo nucifera* Gaertn.) is an aquatic eudicot which can regulate the temperature of its floral chamber between 30-36°C against changing ambient temperatures (Seymour and Schultze-Motel, 1996). Although thermogenicity in the sacred lotus receptacle has been known for more than 100 years (Miyake, 1898) there is still uncertainty regarding heating in the surrounding stamens and petals. Heating has been suggested to occur in both the receptacle (Vogel and Hadacek, 2004) and stamens exclusively (Skubatz and Haider, 2004) and in all floral parts (receptacle, stamens and petals; Seymour and Schultze-Motel, 1998). The present study had four objectives. Firstly, to clarify if the sacred lotus stamens and petals contribute to floral heating. Secondly, to determine if heating was localised to particular regions of each tissue (receptacle included). Thirdly, we hypothesised that similar to the receptacle (Watling *et al.*, 2006; Grant *et al.*, 2008; Chapter 2), the petals and stamens would use the alternative

pathway to generate heat. Our final objective was to identify if carbohydrates or lipids were the thermogenic substrate in these tissues.

MATERIALS AND METHODS

Plant material and temperature measurements

Sacred lotus (*Nelumbo nucifera* Gaertn.) flowers were collected from the Adelaide Botanic Gardens, South Australia during consecutive summer seasons 2006-2008. Flowers were grouped into developmental stages 0-4 as described by Grant *et al.* (2008; Chapter 2). Briefly, stage 0 is a pre-thermogenic closed bud and stages 1-3 are thermogenic, with maximum heating at stage 2 (also see Chapter 2 Fig. 2.1 & Table 2.1). Stamens and petals are only present during stages 0-3. At stage 4, the petals and stamens abscise, leaving a post-thermogenic receptacle which becomes a photosynthetic seed bearing organ (Miller *et al.* 2009). The temperature of the intact receptacle, stamens and petals were measured in the field using an infrared thermometer (Scotchtrack T Heat tracer IR1600L; 3M, Austin, TX, USA) and again 30 s after removal from the pedicel. Temperature measurements for the petals were taken towards the basal end. In addition, the temperatures of a nearby non-thermogenic bud and leaf were measured for comparison. Immediately after temperature measurements the floral tissue was returned to the lab on ice for mitochondrial isolation. Sections were also frozen at -80°C for lipid and carbohydrate analysis. In January 2009 thermal images of intact stage 2 flowers and of excised floral parts were also obtained to illustrate the spatial distribution of temperature throughout the flower parts. Thermography (ThermaCAM S65, Flir Systems, Lindfield NSW) was also used to measure heat loss from recently excised heating tissues and equivalent non-heating tissues. Receptacle, stamen and petal tissues were "killed" by freezing, then heated in a water bath to the equivalent temperatures observed on the flower. The rate of cooling was compared to excised, live thermogenic tissues over an equivalent range with similar background air temperatures.

Respiration and discrimination analysis

Discrimination during respiration and steady state flux of electrons through the cytochrome c and alternative pathways of stamens and petals at each developmental

stage was determined using the on-line oxygen isotope technique described in Watling *et al.*, (2006). Briefly, respiration rates and differential uptake of oxygen stable isotopes were measured simultaneously in six sequential samples taken from the gas phase surrounding the respiring tissues. The isotopic discrimination factors (D) and partitioning of electrons between the cytochrome and alternative pathways were calculated essentially as previously described. The r^2 of all unconstrained linear regressions between –ln *f* and ln (R/R_o), with a minimum of six data points, was at least 0.992. To establish the discrimination endpoints for the alternative (Δa) and cytochrome (Δc) oxidases, stamens and petals were infiltrated with either 16 mM KCN or 25 mM SHAM (made from a 1M stock solution in 0.05% DMSO) prior to measurement. The endpoints for stamens (Δc = 17.5 ± 1.0‰, and Δa = 25.6 ± 1.8‰; mean ± sd) and petals (Δc = 16.3 ± 1.1‰, and Δa = 26.6 ± 2.7‰; mean ± sd) were then used to calculate the flux through the alternative and cytochrome pathways in uninhibited tissues as described in Ribas-Carbo *et al.* (2005).

Mitochondrial isolation and immunoblots

Isolation of washed mitochondria was based on the method of Day *et al.* (1985) with minor modifications as described by Grant *et al.* (2008; Chapter 2). Approximately 10 g of petals and 5 g of stamens were used for each isolation. In addition to whole tissue isolation, the petals and stamens were separated into three sections; top, middle and base for the petals, and appendage (distil), anther sacs (medial) and filament (proximal) for the stamens. Receptacle mitochondria for AOX activation analyses were isolated from 40 g of stage 1 receptacles. Each receptacle was divided into three sections; dermal, upper mesenchyma and basal mesenchyma. Firstly, the epidermal and hypodermal layers of the receptacle were removed and pooled (dermal section). This left a spongy inner core of glandular mesenchyma (Vogel and Hadacek, 2004) which was bisected horizontally into upper and basal mesenchyma sections. Crude mitochondria isolated from these three sections were further purified using a three-step Percoll gradient. The washed mitochondria were placed on the gradient (Percoll 50% [v/v], 35% [v/v] and 20% [v/v]) in buffer (250 mM sucrose, 10 mM HEPES-KOH (pH 7.2), 0.2% [w/v] fatty acid free BSA) and spun for 1 h at 20 000 g and 4 °C. Collected mitochondria were washed (0.4 M mannitol, 10 mM MOPS/KOH (pH 7.2), 0.1% [w/v] fatty acid free BSA) twice by centrifugation at 10 000 g and the final pellet re-suspended in 1 ml wash buffer. The protein concentration of the mitochondria was

determined using the Bradford (1976) method. Separation of proteins by SDS-PAGE electrophoresis, transfer to PVDF membrane and detection of AOX protein with the monoclonal antibodies raised against *Sauromatum guttatum* AOX (Elthon *et al.*, 1989b), Anti-COXII (Agrisera) raised against subunit II of cytochrome c oxidase, a monoclonal antibody reacting with porin (PM035, Dr T Elthon, Lincoln, NE, USA) and pUCP antibodies raised against *Symplocarpus foetidus* (Ito, 1999) and soybean (Considine *et al.*, 2001) were performed as previously described (Grant *et al.*, 2008; Chapter 2).

AOX capacity

AOX capacity was measured in isolated mitochondria from sacred lotus receptacle sections; dermal, upper mesenchyma and basal mesenchyma. A Clark type electrode was used to measure O_2 uptake at 25^{o}C in 1.8 ml of incubation buffer (0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM MOPS/KOH (pH 7.2), 0.1% [w/v] fatty acid free BSA). The O_2 concentration in air-saturated buffer at 25^{o}C was estimated at 250 μM in each experiment. Mitochondrial activity was initiated with the addition of 2 mM NADH and 20 mM succinate to the cuvette. AOX capacity was calculated as the KCN-insensitive and *n*-propyl gallate-sensitive O_2 consumption.

Soluble carbohydrate and starch assay

Soluble carbohydrates and starch were determined in stamen tissue from stages 0, 2 and 3 using the methods of Scholes *et al.* (1994) and Caporn *et al.* (1999) as previously described (Grant *et al.*, 2008; Chapter 2). Stages 0, 2 and 3 represent pre-thermogenic, thermogenic and post-pollen stages, respectively.

Fatty acid analysis

Total lipid was extracted from 0.4 g of frozen tissue using standard methods (Folch *et al.*, 1957) as described by Cyril *et al.* (2002). The tissue was ground to a fine powder in liquid nitrogen (N_2) using a mortar and pestle and then further homogenised with 10 ml of ultra pure chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.01%, w/v) as an antioxidant. Total lipids were separated into triacylglycerides (TAG - neutral lipids) and phospholipids (PL - charged lipids), by sequential elution in hexane and ethyl acetate from Sep-Pac silica columns (Waters, Milford, MA, USA). PL and TAG were trans-methylated using the method of Lepage and Roy (1986). The fatty acid

methyl esters were separated by gas-liquid chromatography on a Shimadzu GC 17A (Shimadzu, Sydney, Australia) with a Varian WCOT Fused Silica Column (50 m x 0.25 mm ID, CP7419, Sydney, Australia). Fatty acids were identified using retention times of an external standard (FAME Supelco, Bellefonte, PA, USA). The samples were quantified against heneicosanoic acid (21:0) as the internal standard (Sigma Aldrich, Sydney, Australia) so that fatty acids could be expressed as an absolute amount as well as a percentage of the total lipid present in the sample. The stages collected for fatty acid analysis were stage 0 (pre-thermogenic), stage 2 (thermogenic) and stage 4 (post-thermogenic) in the receptacle and stage 0 (pre-thermogenic), stage 2 (thermogenic) and stage 3 (post-pollen) in the stamens.

Statistical analysis

Mean differences in relative AOX and COX protein, AOX capacity, floral temperature changes, starch, soluble carbohydrates and fatty acids were investigated using one-way ANOVA and paired T-tests. Where significant differences were identified *post hoc* Tukey-HSD tests were performed. Data were tested for normality and homogeneity of variances using Shapiro-Wilk W and Bartlett's tests, respectively. Stamen and petal AOX/porin protein and stamen sucrose determination data were log transformed to satisfy the assumptions of ANOVA. All statistical analyses were carried out using JMP 5.1 (SAS Institute Inc, Cary, NC, USA). Significant differences at $P<0.05$ are reported.

RESULTS

Do sacred lotus stamens and petals heat?

Whilst intact and attached to the pedicel it was difficult to observe whether the petals and stamens heated independently of the receptacle (Fig. 4.1A). Therefore, floral parts of stage 2 flowers (see Chapter 2 for description of stages) were removed and separated (Fig. 4.1B). Immediately after removal from the pedicel, parts of stage 2/3 flowers were significantly warmer than non-thermogenic leaf tissue with the receptacle heating the most, the petals the least and the stamens heating intermediately (Table 4.1; $F_{3,32}= 19.28$, $P<0.0001$). Temperature change was measured for 3 min after the floral parts were separated from the pedicel. For all floral parts, thermogenic tissues (i.e. fresh and heating) lost significantly less heat than the equivalent non-heating tissue (tissue which had been frozen; Table 4.1; $t= 4.53$, $P=0.02$). This was most pronounced in the non-

heating receptacle which lost heat 8-fold faster than the heating receptacle, but heat loss was also 3-4-fold faster in non-heating stamens and petals than the equivalent heating tissue (Table 4.1). Heat loss from heating tissues was lowest in receptacles (0.12°C in per minute) intermediate in petals (0.50°C per minute) whilst stamens had the largest temperature loss at 0.73 °C per minute (Table 4.1). Heating in the petals was predominantly towards the base (Fig. 4.1B) while all sections of the stamens (filament, anther sacs and appendage) remained warm (Fig. 4.1B). Closer inspection revealed that the filaments of the stamens were somewhat warmer than the distal sections (Fig. 4.1C). Attached stamens also remained 10°C higher than ambient temperature 4 min after the receptacle had been removed from the flower (see supplementary material Fig S4.1 page 83).

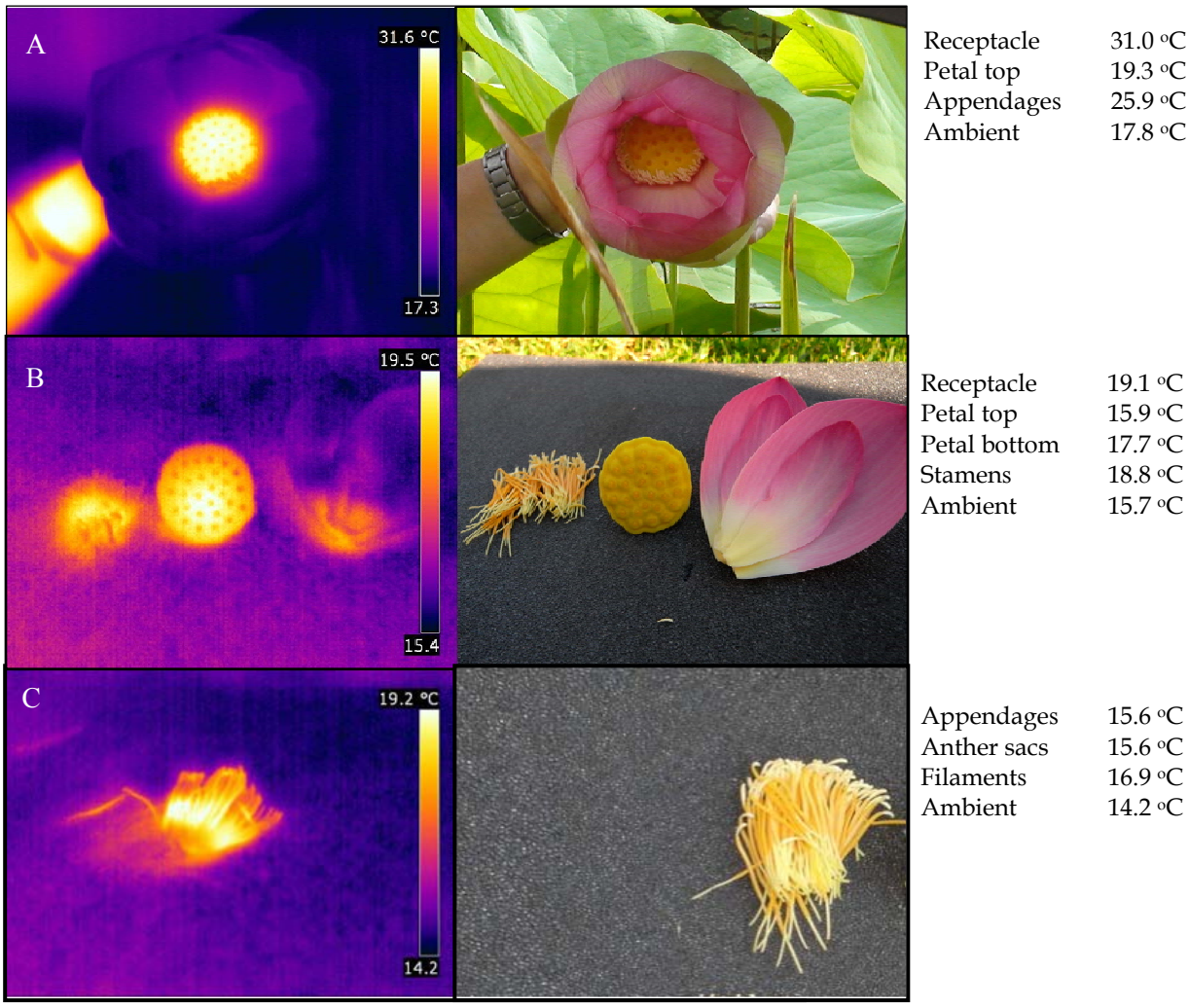

Figure 4.1. Thermal image (left) and photograph (right) of stage 2 sacred lotus floral tissue. A) whole flower (intact and *in situ*), B) dissected flower parts; stamens (left), receptacle (middle) and petals (right), C) detached stamens; appendage (top), anther sacs (middle) and filament (bottom). Please note that the photograph images are slightly offset from the thermal images.

Table 4.1. Heating in thermogenic and non-thermogenic sacred lotus floral tissue.
Flowers in stage 2 were collected between 7.00 and 8.00am AEST. For thermogenic tissue, flowers were cut from the pedicel, separated into tissue types (receptacle, petals and stamens) and placed on foam. Time lapse thermal images were taken every 30 sec for 3 minutes. For non-heating tissue, flowers were collected and frozen at -80 °C. The tissue was thawed and heated in a water bath to 34 °C, placed on foam and time lapse thermal images taken. Where shown, rows with different letters are significantly different from each other (data points are means ± sd of *n*=10 samples).

Tissue	Amount of heating above non-thermogenic tissue immediately after removal from pedicel (°C)	Temperature decrease per min (^{o}C)	
		Thermogenic tissue	Equivalent non-heating tissue
Receptacle	8.1 ± 1.9^{a}	0.12	1.05
Stamens	6.8 ± 2.0^{ab}	0.73	2.09
Petals	2.8 ± 4.2^{b}	0.50	2.52
Leaf	-1.9 ± 3.3^{c}	na	na

Respiratory flux and proteins in the stamens

Total respiratory flux was high in stamens reaching a mean maximum 0.029 ± 0.005 μmol O_2 g fw^{-1} s^{-1} (± se) in stage 3 stamens (Fig. 4.2A). There was considerable variation in total respiratory flux of stamens, especially in stage 3 flowers where anthers are dehiscing prior to stamen and petal abscission (range 0.0093 to 0.042 μmol O_2 g $fw^{-1}s^{-1}$; Fig. 4.2A). As a result of this variation there was no significant change in total respiratory flux across developmental stages. There was no detectable AOX flux in pre-thermogenic (stage 0) stamens (Fig. 4.2A), but AOX flux had significantly increased by stage 1, and remained similar across stages 1-3 when, on average, it accounted for 15 to 23% of total respiratory flux ($F_{3,22}$=3.02, P =0.0014). The proportion of total flux via AOX was also highly variable among stamens within a single developmental stage, for example, at stage 2, the proportion of AOX flux ranged from 3 to 40% of total respiratory flux. Flux through the COX pathway averaged 0.020 ± 0.008μmol O_2 g $fw^{-1}s^{-1}$ in stamens and was similar across the development sequence (Fig. 4.2A).

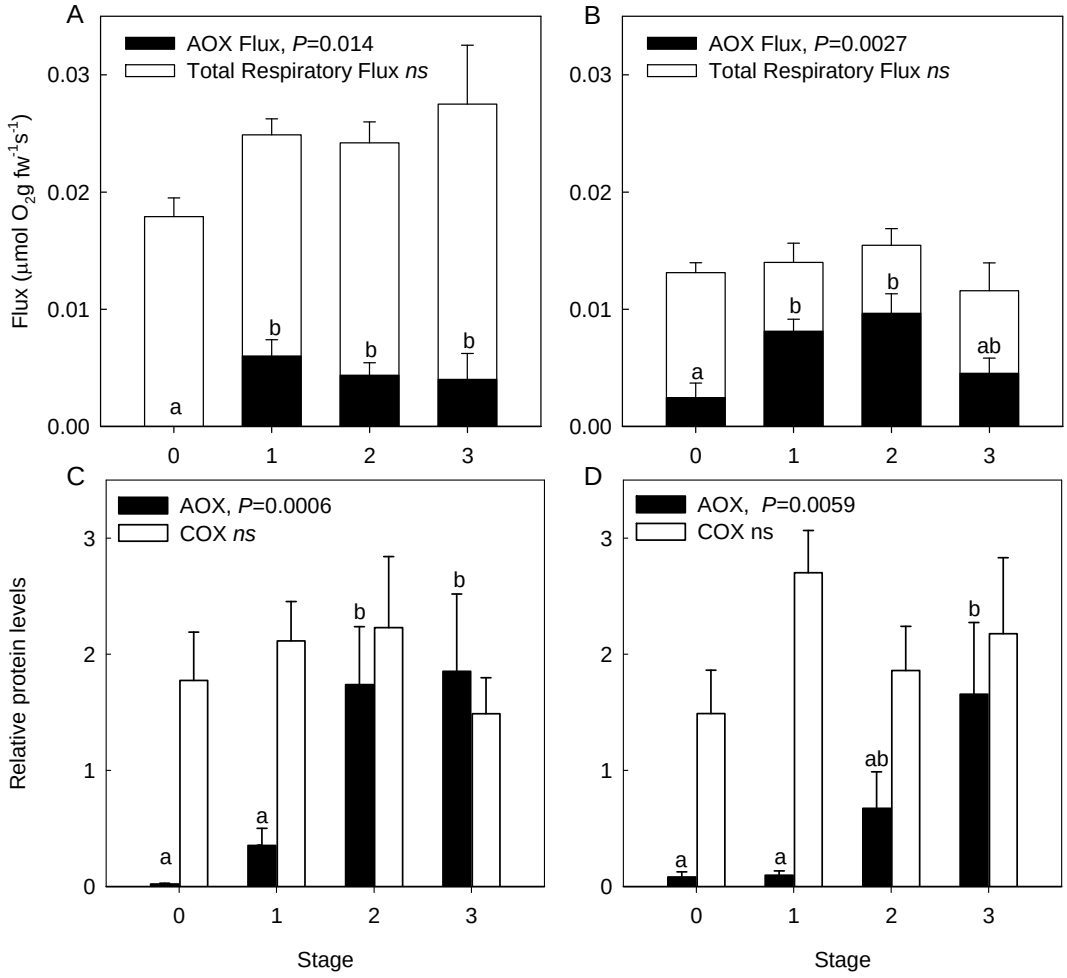

Figure 4.2. Changes in staminal (A & C) and petal (B &D) respiratory fluxes (top) and protein levels (bottom) during floral development. Thermoregulation occurs in the receptacle during stages 1, 2 and 3 and pollen is shed during stage 3. Data are means ± se, *n*=5-10. Columns with different letters are significantly different at $P<0.05$. In A & B the unshaded region is the COX respiratory flux.

AOX was detected in stamens as a ~32 kDa protein. AOX protein levels in the stamens increased until stage 2 and remained high throughout stage 3 (Fig. 4.2C). Stage 2 AOX protein levels were 5-fold higher than stage 1 ($F_{3,16}$=9.96, P =0.0006). COX protein levels in stamens did not change significantly during development (Fig. 4.2C). There was no detection of pUCPs in the stamens at any developmental stage.

Respiratory flux and proteins in the petals

Total respiratory flux in petals was similar at all developmental stages (Fig. 4.2B), with the highest mean flux, 0.015±0.001 µmol O_2 g $fw^{-1}s^{-1}$ measured in stage 2 petals. Similarly, no significant differences were detected in flux through the COX pathway across the developmental sequence (Fig. 4.2B). AOX flux in the petals increased significantly with the onset of thermogenesis, increasing more than 3-fold from stage 0

to stage 1 (Fig. 4.2B; $F_{3,23}$=4.53, *P* =0.012). Mean AOX flux then declined by 53% from stage 2 to stage 3, however this decrease was not significant.

AOX in the petals was also detected as a ~32 kD protein. AOX protein levels in petals remained low during stages 0 and 1 but increased 5- and 12-fold from these initial stages to stages 2 and 3, respectively (Fig. 4.2D). AOX protein levels increased significantly within the thermogenic period with stage 3 petals containing the largest amount of AOX protein (Fig. 4.2D; $F_{2,19}$=7.81, *P*=0.006). Mean COX protein levels did not change significantly across the developmental sequence (Fig. 4.2D). There was no detection of pUCPs in the petals.

AOX protein distribution in stamens, petals and receptacle

Both AOX and COX proteins were distributed evenly throughout the length of the stamens (Fig. 4.3A). By contrast, in the petals there was 10-fold decrease in relative AOX protein from the base to tip (Fig. 4.3B; $F_{3,19}$= 28.56, *P* <0.0001) whilst COX protein levels remained similar along the length of the petal (Fig. 4.3B). AOX protein was detected at a similar level in three parts of dissected sacred lotus receptacles; dermal layer, upper mesenchyma and basal mesenchyma (Table 4.2; $F_{2,16}$=2.12, *P*=0.15). In addition, there was no difference in AOX capacity, measured as cyanide resistant respiration, in mitochondria isolated from the three sections (Table 4.2; $F_{2,19}$=0.79, *P*=0.46).

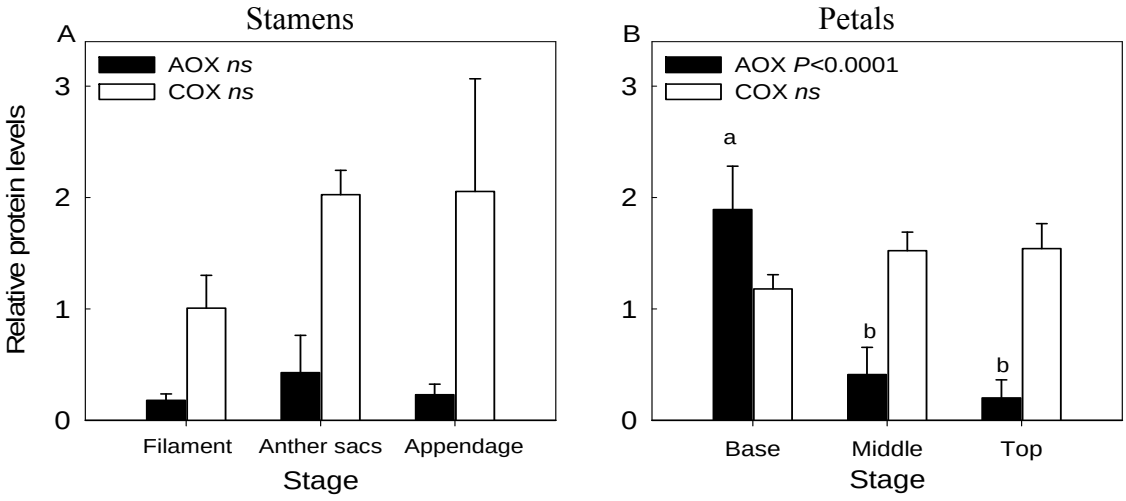

Figure 4.3. Changes in AOX (filled) and COX (open) protein levels in mitochondria isolated from dissected stage 2 sacred lotus stamens (A) and petals (B). Data are means ± se, *n*=5-6 samples. Columns with different letters are significantly different at *P*<0.05 (*ns*= not significant).

Table 4.2. AOX capacity and relative AOX protein levels in mitochondria isolated from dissected sacred lotus receptacles. Each receptacle was cut into three sections; the dermal layer, the upper and basal mesenchyma. AOX capacity was measured as KCN-insensitive and n-propyl gallate sensitive oxygen consumption. The data are means ± se of *n*=6-8 assays and were not significantly different between sections. Tissue was collected from flowers in stage 1 and 2 of the thermoregulatory period

Receptacle section	AOX capacity (nmol O_2 min^{-1} mg^{-1} protein)	Relative AOX protein
Epidermis	214 ± 11	7.8 ± 0.5
Upper mesenchyma	267 ± 13	5.8 ± 0.4
Basal mesenchyma	248 ± 11	7.9 ± 0.5

Contribution of parts to whole flower heating

Based on AOX flux and tissue mass we calculated the contribution of each of the floral parts to the AOX flux of the whole flower. In the receptacle AOX flux is directly correlated with heat production (Grant *et al.*, 2008; Chapter 2; Watling *et al.*, 2008) and if we assume this relationship holds true for the petals and stamens, the contribution of AOX flux for each tissue will be directly related to the amount of heat produced by that part. During the thermogenic period (stages 1-3) the receptacle contributes 39-50% of the AOX flux, the petals 43-45% and the stamens 8-16% (Table 4.3). AOX flux in the receptacle increased 22% between stages 1 and 2 and remained high during stage 3. Conversely, in the stamens, the contribution to total floral AOX flux halved between stages 1 and 2 and remained less than 10% of floral AOX during stage 3 (Table 4.3). The contribution made by the petals was constant across the thermogenic period (Table 4.3).

Table 4.3. Contribution of various parts to total floral AOX flux throughout development. AOX flux for each tissue and proportion of floral AOX flux for sacred lotus floral parts receptacle, stamens and petals during the thermogenic stages. Mass (means ± se; *n*= 5-13 samples). Flux data was sourced from Fig. 4.2 and Grant *et al.,* (2008; Chapter 2). N.B. Stamens and petals abscise after stage 3 and receptacle greens.

Tissue	Stage	Mass (g)	AOX flux per tissue (μmol O_2 s^{-1})	% Floral AOX flux
Receptacle	1	6.36 ± 0.70	0.095	39
	2	8.67 ± 0.70	0.217	50
	3	9.69 ± 0.66	0.121	47
Stamens	1	6.54 ± 0.40	0.039	16
	2	6.86 ± 0.43	0.030	7
	3	5.07 ± 0.69	0.020	8
Petals	1	13.79 ± 1.30	0.112	45
	2	19.18 ± 2.18	0.185	43
	3	25.33 ± 4.22	0.115	45

Soluble carbohydrates and starch in staminal tissue

There were significant changes in staminal starch concentrations during floral development (Fig. 4.4A; $F_{2,14}$= 6.78, *P*= 0.0121). Starch concentrations doubled from 8.3 ± 4.2 mg g fw^{-1} to 16.6 ± 3.4 mg g fw^{-1} between stages 0 and 3 (Fig. 4.4A). Concentrations of fructose and glucose remained low until late in floral development (stage 3) when they increased 4-fold (Fig. 4.4C; $F_{2,14}$= 60.27, *P*<0.0001). In contrast, sucrose concentrations decreased by 70% from 6.1 ± 5.0 mg g fw^{-1} to 1.7 ± 0.1 mg g fw^{-1} between stages 0 and 2 (Fig. 4.4B; $F_{2,14}$= 5.12, *P*=0.029), although this decrease was not significant due to the large variances during the pre-thermogenic stage 0. Sucrose concentrations then increased significantly between stages 2 and 3 to a maximum concentration of 9.3 ± 2.4 mg g fw^{-1} (Fig. 4.4B).

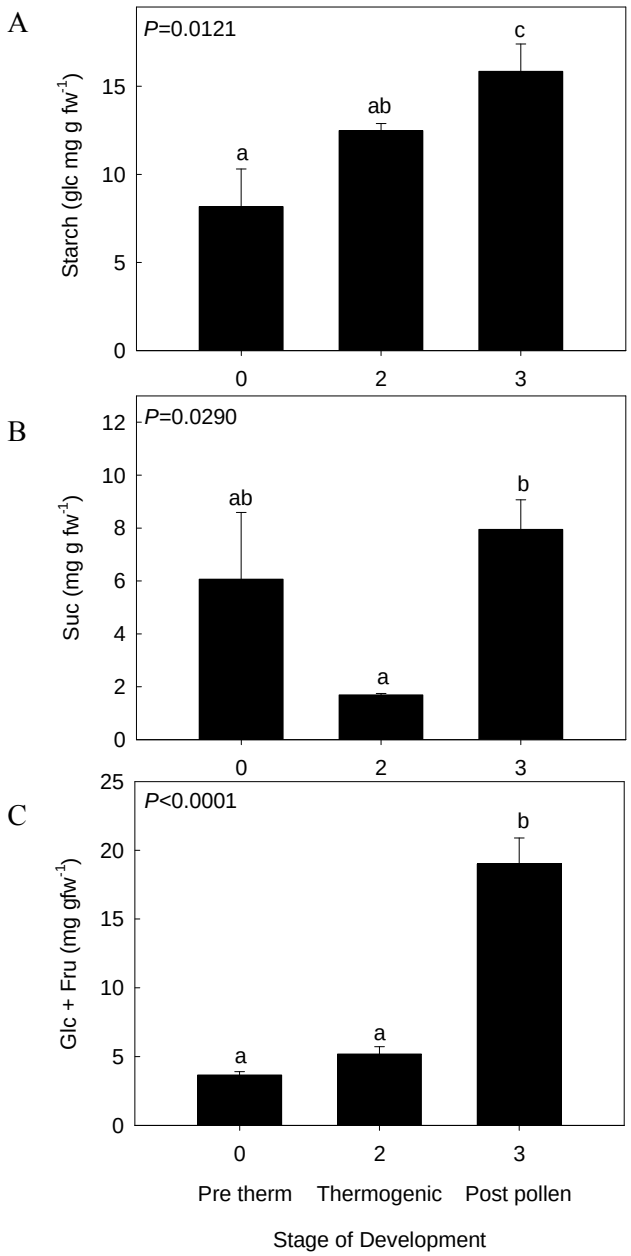

Figure 4.4. Changes in concentration of starch (A), sucrose (Suc) (B) and glucose (Glc) & fructose (Fru) (C) in sacred lotus stamens during floral development. Columns are means ± se (n=5). Columns with different letters are significantly different at P<0.05.

Lipid content and composition of the receptacle and stamens

Total lipid content of the sacred lotus receptacles remained similar with a mean (± se) of 1.49 ± 0.10 mg g fw^{-1} during floral development (Fig. 4.5A). Total lipids were comprised of 55-64% phospholipids (PL) and 36-45% triacylglycerides (TAG; Fig. 4.5A). Phospholipid content in sacred lotus receptacles also remained similar (range 0.80 ± 0.04 to 1.04 ± 0.09 mg g fw^{-1}) during floral development. Triacylglycerides did not change from stage 0 to stage 2, but significantly decreased (40%) to 0.46 ± 0.03 mg g fw^{-1} in stage 4 receptacles (post-thermogenesis; $F_{2,14}$ =7.1237, P=0.0091). Total lipids

(TAG and PL) in stamens were more than 2-fold higher than in the receptacle tissue and remained high during floral development with a mean of 3.72 ± 0.17 mg g fw^{-1} (Fig. 4.5B). There were no significant changes in staminal total PL (46 ± 1.9%) or TAG (54 ± 1.9%) during development (Fig. 4.5B).

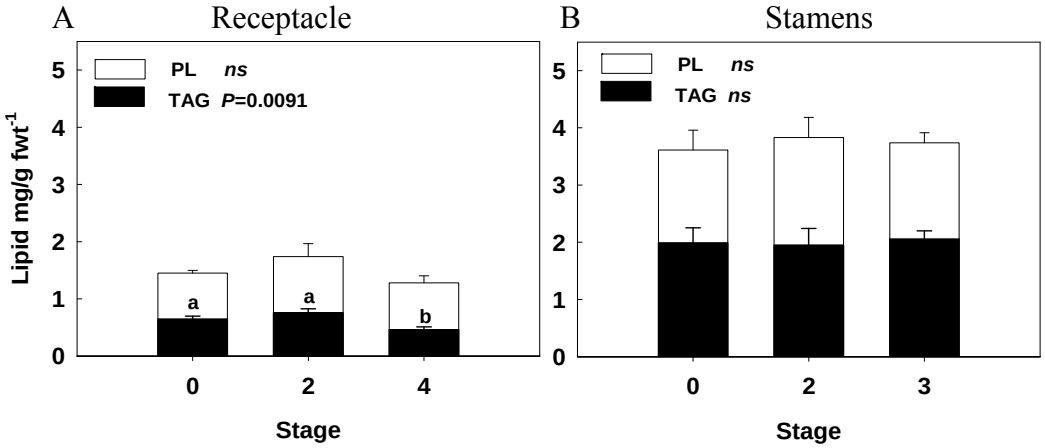

Figure 4.5. Total lipid composition of A) receptacle and B) staminal tissue during floral development shown as PL (phospholipids) and TAG (triacylglyceride) fractions. Columns are means ± se, n=5. Different letters denote significant differences at $P<0.05$, *ns*= not significant. Individual components are shown in Supplemental Table 4.1

Compositionally, for both the receptacle and stamens, fatty acids were dominated by palmitic (16:0), linoleic (18:2) and linolenic acids (18.3; Supplemental Table 4.1 page 84). Staminal TAG was the only measured lipid fraction that contained lignoceric acid (24:0), which increased 7-fold from 1.4% to 10.0% during floral development (Supplemental Table 4.1 page 84). The ratio of carbohydrate to lipids was high, with 5 to 10 fold more carbohydrates than lipids per gram fw for all stages in both stamens and receptacles (carbohydrate data for receptacle are from Grant *et al*., 2008; Chapter 2).

DISCUSSION

Although it is clear that the sacred lotus receptacle heats via the alternative oxidase (Watling *et al.*, 2006; Grant *et al.*, 2008; Chapter 2) there is some debate in the literature concerning the role of stamens and petals in floral heat production. Several workers have suggested that the stamens are the predominant heating tissue (Schneider and Buchanan, 1980; Skubatz *et al.*, 1990), however other work based on metabolic rates, reports that the receptacle contributes 50% of the floral heating and the stamens and petals 25% each (Seymour and Schultze-Motel, 1998). The only study to investigate direct temperature measurements found no evidence of staminal heating and implied that heating in the petals and stamens was only a result of heat transfer from the thermogenic receptacle (Vogel and Hadacek, 2004). This study presents a range of data which together confirm that in addition to the receptacle, the surrounding stamens and petals of the sacred lotus also heat.

Temperatures of both petals and stamens were significantly higher than the non-thermogenic leaves and although some heat was lost on removal from the flower, sufficient remained to suggest that these organs may heat independently. Further evidence is provided by thermal images showing floral parts separated from the pedicel and each other, where stamens and petals are clearly warmer than their surroundings and stay warm for longer than equivalent non-heating tissue (Fig. 4.1B, Table 4.1). This ability of all parts of newly excised flowers to stay warm (Table 4.1) suggests that they are able to generate heat to replace that which is lost. As expected, heat loss was most rapid in the small, spindly stamens and least in the denser receptacles (Table 4.1). Further evidence for independent heating of stamens is seen in the thermograph (Supplemental Fig. 4.1) where upon the removal of the receptacle from the flower, the attached stamens remained nearly 10°C higher than ambient temperature for 4 min. Whilst necessary to prevent heat transfer from the receptacle, removing the petals and stamens from the flower could also reduce heating by disturbing metabolism or inducing a wound response. However, this is not always the case since in the thermogenic *Philodendron bipinnatifidum*, for example, sterile and fertile male florets continue to heat for up to 30 hours after removal from the plant (Chapter 5). Although Vogel and Hadacek (2004) reported no staminal heat production, they used flowers which appeared to be smaller, have fewer petals and, on average, were 2°C cooler than

the ones used in the present study. The size of the heating tissue has been shown to influence the amount of heating, with larger spadices of *P. bipinnatifidum* and skunk cabbage (*S. foetidus*) regulating at higher temperatures than smaller spadices (Nagy *et al.*, 1972; Seymour and Blaylock, 1999). It may also be the case that different populations of *N. nucifera* have different heating patterns as was found for the thermogenic skunk cabbage, with one group heating to approximately 4°C and another group averaging 17°C above ambient (Seymour and Blaylock, 1999).

An increase in flux through the AOX pathway was seen in both stamens and petals with the onset of thermogenesis (Fig. 4.2). Total respiratory flux was 40% higher in the stamens than the petals, however this is not surprising considering the high-energy demand of pollen development. *Lilium longiflorum* pollen respires 10 times faster than vegetative tissue (Dickinson, 1965) and electron micrographs of *N. nucifera* stamens showed high numbers of mitochondria (Skubatz *et al.*, 1990). AOX and total respiratory flux in the receptacle was more than double that of the stamens or petals (Grant *et al.*, 2008; Chapter 2) supporting the view that the receptacle is the predominant heating tissue. Previously, total respiratory rates were used to determine the role of the floral parts in whole flower heating with the receptacle responsible for 50% and both petals and stamens 25% (Seymour and Schultze-Motel, 1998). Here, we have used only AOX respiratory flux to estimate the amount of heat produced by the various floral tissues. Our results suggest that the petals contribute a relatively constant 45% of the heat, matching that contributed by the receptacle, whilst stamens contribute up to 16% in stage 1 and 8% or less during stages 2 and 3 (Table 4.3). This indicates an important role for the petals in floral heating rather than the trivial role previously proposed (Schneider and Buchanan, 1980; Skubatz *et al.*, 1990; Vogel and Hadacek, 2004). Supporting this, a recent study demonstrated the importance of petals for reproductive success in *N. nucifera* (Li and Huang, 2009). Seed set from hand pollinated *N. nucifera* flowers without petals, was significantly lower than that of intact flowers (Li and Huang, 2009). Considering that *N. nucifera* petals contribute approximately 45% of floral heat, this suggests that the role of heating in this species may not be predominantly to attract insect pollinators as previously thought (Seymour and Schultze-Motel, 1998), but a mechanism to provide the optimum temperature for reproductive success. In most thermogenic species heating is associated with reproductive floral parts rather than non-reproductive parts such as the petals.

Furthermore, most explanations for the evolution of thermogenesis focus on the attraction of insect pollinators to these reproductive parts (Meeuse, 1975; Seymour and Schultze-Motel, 1998), which may be the reason heating of the petals has been overlooked.

The increase in AOX flux in stamens and petals was followed by an increase in AOX protein, which reached a maximum in stage 2 stamens and stage 3 petals. The build-up of AOX protein in each tissue was not synchronised within the flower, but it closely preceded reproductive maturity of the stigma in the receptacle (Grant *et al.*, 2008; Chapter 2) and pollen in stamens (Fig. 4.2) indicating a possible role of thermogenesis in reproductive maturity. In the petals, AOX protein was found in the base of the petal, where the petal tissue becomes yellow and spongy similar to the receptacle. This is also where the bulk of the heating occurs, as shown by thermal images (Fig. 4.1). If heating in the sacred lotus is important for the attraction of invertebrate pollinators (Seymour, 2001a), the presence of AOX in the base of the petals and associated heat may assist to lure insect pollinators towards the bottom of the floral chamber to the mature pollen. Alternatively heating in all parts of the flower may be important in maintaining the correct temperature for floral development. Recently it was shown that low temperatures during the fertilisation period significantly decreased seed set in *N. nucifera*, indicating that thermogenesis in this species may indeed be important for successful pollen germination or pollen tube growth (Li and Huang, 2009). Specific temperatures have been shown to increase pollen performance in a number of different plant species (Galen and Stanton, 2003; Van Der Ploeg and Heuvelink, 2005; Maeda *et al.*, 2008), with unfavourable temperatures usually leading to low germination. COX protein and flux through the cytochrome pathway remained constant during development and heating stages in all flower parts and plant uncoupling proteins (pUCP) were not detected (data not shown), providing further evidence that pUCPs are not likely to be involved in thermogenesis in the sacred lotus (Watling *et al.*, 2006; Grant *et al.*, 2008; Chapter 2).

Heating in the sacred lotus receptacle has been attributed to flux through the alternative pathway (Watling *et al.*, 2006; Grant *et al.*, 2008; Chapter 2), however the localisation of the AOX protein within the receptacle remained unclear. Here we report that throughout the sacred lotus receptacle the AOX protein is distributed evenly and the

activity of mitochondria is uniform (Table 4.2). In many thermogenic species AOX is localised within specific tissues, for example localisation of AOX in petals and pistil but not the anthers or filaments in the spadix of the skunk cabbage (Onda *et al.*, 2008), and in the male and sterile florets but not the female florets in *P. bipinnatifidum* (Chapter 5). In the lotus, AOX is evenly spread through both the male and female floral tissue suggesting the capacity to heat is ubiquitous. Sacred lotus receptacles contain two isoforms of AOX (Grant *et al.*, 2009; Chapter 3) and it maybe that only one of these isoforms is active in thermogenesis. Expression studies are needed to determine which isoform(s) of AOX are present in stamen and petal tissues.

A large supply of energy is needed to fuel thermoregulation in plants and metabolic rates in these species may equal that of similar sized animals (Lamprecht *et al.*, 2002b). Both carbohydrates (*A. maculatum*) and lipids (*P. bipinnatifidum*) have been found to fuel thermogenesis (ap Rees *et al.*, 1977; Walker *et al.*, 1983). Starch and soluble carbohydrate levels in the stamens showed similar patterns to that in the receptacle during development, although soluble carbohydrate levels in the stamens (Fig. 4.4) were double that of the receptacle (Grant *et al.*, 2008; Chapter 2). The use of sucrose for thermogenesis could account for the 60% decrease in sucrose in the stamens on the onset of thermogenesis in stage 2 (Fig. 4.4). However we cannot attribute all changes in stamen carbohydrates with thermogenesis, as pollen development and maturation are taking place. After pollen maturation, an increase in soluble sugars and starch was reported in *Lilium* stamens (Clement *et al.*, 1996). This was also seen in sacred lotus stamens with both soluble sugars and starch increasing post-pollen release in stage 3 (Fig. 4.4). This may be because the remaining appendage and filament act as sink organs once anther maturation is complete (Clement *et al.*, 1996).

There was little change in total lipids in receptacle and staminal tissue. A 40% decrease in total receptacle TAG between stage 2 and 4 (peak and post-thermogenesis; Fig. 4.5) could indicate that lipids play a role in providing energy for thermogenesis in the receptacle. However, this decrease was not as prominent as the 89% decrease in starch in the receptacle between peak and post-thermogenesis (Grant *et al.*, 2008; Chapter 2). Also, these tissues contained at least 5-fold more carbohydrate than lipid per gram fresh weight, suggesting that starch is the major source of fuel for thermogenesis in the sacred lotus flower. A similar carbohydrate/lipid ratio was reported in thermogenic *Magnolia*

tamaulipana flowers (Dieringer *et al.*, 1999) where high carbohydrate values were attributed as a food supply for visiting invertebrates. However, in the lotus there was no evidence of invertebrate damage to floral tissue. The proportions of PL and TAG in sacred lotus floral tissues are similar to that found in the flower bud of cotton plants (*Gossypium sp*.; Thompson *et al.*, 1968).

There were, however, compositional changes in the polar (PL) and non-polar (TAG) lipids (Supplemental Table 4.1). The increase in linolenic acid (18:3) during development may reflect the greening of the organ as it becomes photosynthetic after petals and stamens abscise (Miller *et al.*, 2009). Increases in linolenic acid (18:3) with greening of leaves have been shown in a number of higher plants including barley (Appelqvist *et al.*, 1968), *Ligustrum ovatifolium* and *Vicia faba* (Crombie, 1958). The fatty acid 24:0 (lignoceric acid) was only found in the staminal TAG fraction and although it is classified as a minor plant fatty acid (Hitchcock and Nichols, 1971), it is not uncommon. The amount of lignoceric acid increased with floral development and thermogenesis (Supplemental Table 4.1). Although it was not detected in lotus receptacle, it has been reported in the female parts of other plants, for example in the stigma of *Nicotiana tabacum* (Cresti *et al.*, 1986). Without double bonds or other functional groups this long chain fatty acid is nearly chemically inert and thus may remain unchanged when subjected to high temperatures. The thermophile fungi *Aspergillus fumigatus* contains lignoceric acid (Habe *et al.*, 2008) as do sunflower seeds grown at elevated temperatures (Izquierdo and Aguirrezabal, 2008). However in sunflower seeds there was a decrease in long chain fatty acids, including lignoceric acid, with increased growth temperature indicating that the role of lignoceric acid in thermo-tolerance remains unclear.

Conclusion

We provide evidence that the stamens and petals of the sacred lotus produce heat independently of the thermogenic receptacle. The petals and receptacle contribute the bulk of floral heat throughout floral development whilst the contribution of the stamens is highest during stage 1 suggesting that heating is linked to reproductive maturity of the two sexual tissues. Our data for AOX flux, AOX protein and substrates are consistent with AOX being the sole source of heating in sacred lotus flowers.

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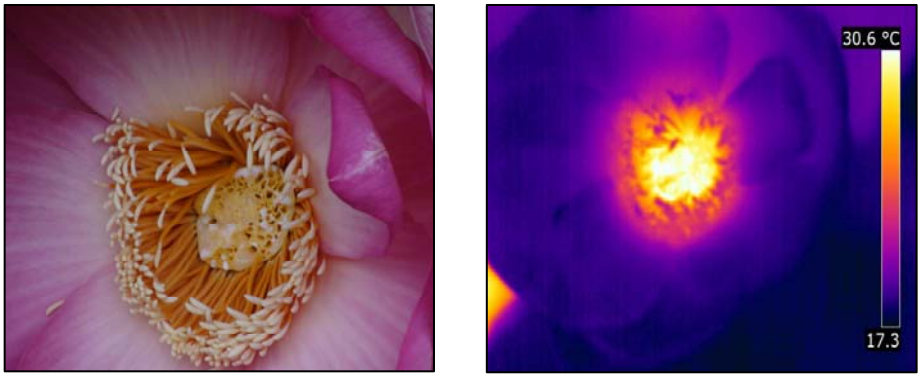
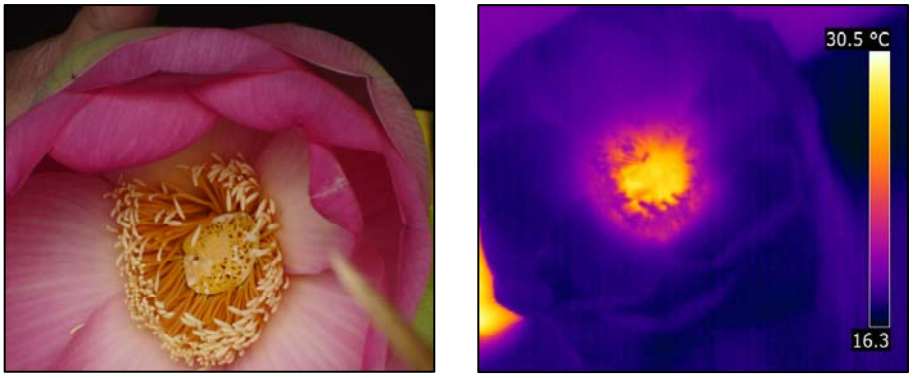
SUPPLEMENTAL MATERIAL

Supplemental Figure 4.1. Photograph (left) and thermal image (right) of attached sacred lotus stamens immediately (upper panel) and 4 minutes after (lower panel) removal of the receptacle.



Stamens 28.2 °C
Ambient 14.3 °C

Flower imaged directly after receptacle removal



Stamens 24.2 °C
Ambient 14.3 °C

Flower imaged 4 min after receptacle removal

Supplemental Table 4.1. Fatty acid content (percent of total) in sacred lotus receptacle and stamens tissue during floral development. Both phospholipid (PL) and triacylglycerides (TAG) fractions were analysed. Data are means ± sd *n*=5. Entries within rows with different letters are significantly different, ns = no significant difference.

	Fraction	Fatty acid	Percent of total (% ± sd)			Analysis of variance	
			Pre-thermogenic (stage 0)	Thermogenic (stage 2)	Post-thermogenic (stage 4)	*P*-value	$F_{2,14}$ stat
Receptacle	PL	16:0 (palmitic acid)	34.6 ± 3.3	30.7 ± 0.9	32.5 ± 1.3	ns	4.1
		18:0 (stearic acid)	5.8 ± 0.5	4.7 ± 1.2	5.8 ± 1.2	ns	1.6
		18:1 (oleic acid)	7 ± 1.0	7.4 ± 0.5	7.6 ± 0.8	ns	0.6
		18:2 (linoleic acid)	32.6 ± 2.5^{a}	30.4 ± 1.1^{ab}	28.1 ± 0.5^{b}	0.008	8.3
		18:3 (linolenic acid)	14.6 ± 2.6 ^{a}	22.6 ± 1.7^{b}	20 ± 1.4^{b}	0.0004	18.7
	TAG	16:0 (palmitic acid)	28.9 ± 0.6^{a}	24.3 ± 2.0^{b}	31.4 ± 2.0^{a}	0.0001	21.8
		18:0 (stearic acid)	8.4 ± 2.7^{a}	3.9 ± 0.5^{b}	7.0 ± 2.7^{ab}	0.019	5.8
		18:1 (oleic acid)	3.9 ± 2.1^{a}	7.4 ± 0.8^{b}	7.1 ±0.8^{b}	0.004	9.5
		18:2 (linoleic acid)	44.6 ± 4.0^{a}	38.5 ± 2.7^{b}	30.2 ± 2.1^{c}	0.0001	26.8
		18:3 (linolenic acid)	8.4 ± 8.3^{a}	23.6 ±1.7^{b}	22.1 ± 3.6^{b}	0.0017	12.0

			Pre-thermogenic (stage 0)	Thermogenic (stage 2)	Post-pollen (stage 3)		
Stamens	PL	16:0 (palmitic acid)	30.2 ± 2.2^{a}	34.4 ± 1.1^{b}	35.3 ± 1.3^{b}	0.0013	12.9
		18:0 (stearic acid)	2.9 ± 0.3^{a}	2.3 ± 0.2^{b}	2.4 ± 0.4^{ab}	0.0298	4.9
		18:1 (oleic acid)	3.7 ± 0.7	2.6 ± 0.5	3.5 ± 0.7	ns	
		18:2 (linoleic acid)	36.7 ± 3.3^{a}	21.6 ± 1.2^{b}	21.9 ± 0.9^{b}	0.0001	76.3
		18:3 (linolenic acid)	22.8 ± 3.1^{a}	33.3 ± 0.5^{b}	31 ± 2.4^{b}	0.0001	24.5
	TAG	16:0 (palmitic acid)	25.2 ± 5.7^{a}	36.0 ± 0.9^{b}	35.2 ± 1.5^{b}	0.0005	15.2
		18:0 (stearic acid)	3.3 ± 0.8^{a}	2.4 ± 0.1^{b}	2.4 ± 0.4^{ab}	0.0298	4.8
		18:1 (oleic acid)	6.1 ± 1.5	4.1 ± 0.3	5.1 ± 1.4	ns	
		18:2 (linoleic acid)	44.3 ± 3.3^{a}	23.4 ± 0.9^{b}	22.3 ± 2.4^{b}	0.0001	125.6
		18:3 (linolenic acid)	15.2 ± 0.8^{a}	22.2 ± 1.9^{b}	17.8 ± 1.5^{a}	0.0001	28.5