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RESEARCH ARTICLE

Floral thermal biology in relation to pollen thermal performance in an early spring flowering plant

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ABSTRACT

- The floral microenvironment impacts gametophyte viability and plant–pollinator interactions. Plants employ mechanisms to modify floral temperature, including thermogenesis, absorption of solar radiation, and evaporative cooling. Whether floral thermoregulation impacts reproductive fitness, and how floral morphological variation mediates thermoregulatory capacity are poorly understood.
- We measured temperature of the floral microenvironment in the field and tested for thermogenesis in the lab in early spring flowering *Hexastylis arifolia* (Aristolochiaceae). We evaluated whether thermoregulatory capacity was associated with floral morphological variation. Finally, we experimentally determined the thermal optimum and tolerance of pollen to assess whether thermoregulation may ameliorate thermal stress to pollen.
- Pollen germination was optimal near 21 °C, with a 50% tolerance breadth of ~18 °C. In laboratory conditions, flowers exhibited thermogenesis of 1.5–4.8 °C for short intervals within a conserved timeframe (08:00–09:00 h). In the field, temperature inside the floral tube often deviated from ambient – floral interiors were up to 4 °C above ambient when it was cold, but some fell nearly 10 °C below ambient during peak heat. Flowers with smaller openings were cooler and more thermally stable than those with larger openings during peak heat. Thermoregulation maintained a floral microenvironment within the thermal tolerance breadth of pollen.
- Results suggest that *H. arifolia* flowers have a stronger capacity to cool than to warm, and that narrower floral openings create a distinct floral microenvironment, enhancing floral cooling effects. While deviation of floral temperature from ambient conditions maintains a suitable environment for pollen and suggests an adaptive role of thermoregulation, we discuss adaptive and nonadaptive mechanisms underlying floral warming and cooling.

INTRODUCTION

As largely sessile organisms, plants often cope with dramatic fluctuations in temperature across their lives. At the reproductive stage in flowering plants, floral temperature can have important consequences for gamete development and viability (Thakur *et al.* 2010; Zinn *et al.* 2010; Hedhly 2011), pollination (Norgate *et al.* 2010), and fertilization (Cerović *et al.* 2000). Plants have evolved a variety of mechanisms that modify floral temperature, including heliotropism (Zhang *et al.* 2010), thermogenesis (Watling *et al.* 2008; Seymour *et al.* 2009), evaporative cooling (Galen 2006), shading of reproductive structures (Karban *et al.* 2023), and the focus or reflection of solar radiation (Kevan 1975; McKee & Richards 1998). While some of these mechanisms (e.g., heliotropism) are likely adaptive (Creux *et al.* 2021), others, such as transpirational cooling, may result from plant-wide physiological responses to ambient temperature that incidentally impact floral temperature. Understanding the variety of mechanisms by which plants alter their floral microenvironment and the impacts of floral temperature on reproductive performance is crucial in the face of rapidly changing temperatures and shifts in phenology that expose plants to extreme thermal conditions.

Gametophyte performance in angiosperms is often highly responsive to the thermal environment (Seymour *et al.* 2009; Rosbakh & Poschlod 2016; Flores-Rentería *et al.* 2018; Heiling & Koski 2024), and is often optimal at intermediate temperatures. For example, in *Argentina anserina* (Rosaceae) pollen exposed to temperatures ranging between 3–39 °C, had optimal germination between 19.5–27.0 °C across populations, and germination was depressed at the 3 and 39 °C extremes (Heiling & Koski 2024). Likewise, Rosbakh & Poschlod (2016) exposed pollen from 21 species to a range of temperatures from 5°–34°C, and the average optimal germination temperature was 21.9 °C (±1.21 SD) across taxa. However, explicit generation of thermal performance curves for gametophyte performance in both crop and wild species is rare (however see Rosbakh & Poschlod 2016; Heiling & Koski 2024), limiting our knowledge of how performance optima and thermal tolerance breadth relate to the range of temperatures experienced in the field during flowering (however see Seymour *et al.* 2009; Rosbakh & Poschlod 2016). Should the temperatures experienced

by flowers fall far from thermal optima for gametophytes, then mechanisms of floral warming or cooling should be beneficial for reproductive performance.

Mechanisms of floral warming are better studied than for cooling (Patiño & Grace 2002; van der Kooi *et al.* 2019), often due to geographic bias in data collection. Floral thermogenesis, the metabolic generation of heat, has been documented in several taxa, especially basal angiosperms (Seymour & Schultze-Motel 1999). Thermogenesis can attract pollinators to a 'heat reward' and enhance the volatilization of floral scents, which also increases pollinator attraction (Seymour *et al.* 2003). The duration of floral thermogenesis has been associated both temporally and spatially with distinct sexual phases of flowers (e.g., thermogenesis only occurs in the male zone of dimorphic *Amorphophallus* especially immediately after anthesis; Claudel *et al.* 2023). Heliotropism is another well-studied mechanism of floral warming in which floral orientation is adjusted to maximize the absorption of solar radiation. Most studies of floral warming focus on the impact on pollinator visitation (e.g., Seymour & Matthews 2006; Dieringer *et al.* 2014; Creux *et al.* 2021), while the relationship between warming and gametophyte viability is less understood.

Evaporative cooling and shading of reproductive structures by petals are the primary known mechanisms of floral cooling. By transpiring water through floral tissues, some plants effectively cool the floral interior (Patiño & Grace 2002; Galen 2006). For example, transpiration has been shown to lower internal flower temperature in both soybean and tobacco (Sinha *et al.* 2022). This process involves opening stomatal apertures on floral tissue while closing stomata on leaf tissue. The effects of evaporative cooling may enhance both seed and pollen performance in some taxa (Patiño & Grace 2002). Recent work has shown that petal positioning over reproductive structures in a suite of Californian taxa can reduce floral temperature, with positive consequences for seed production (Karban *et al.* 2023). Furthermore, some bees have been shown to 'overheat' within warm flowers and take cooling flights (Corbet & Huang 2016). Thus, cooling could increase pollinator visitation and impact reproductive performance. However, our understanding of the incidence of floral cooling is very limited in scope, both geographically and taxonomically (van der Kooi *et al.* 2019).

The capacity for floral warming and cooling is likely mediated by both variation in floral traits and ambient conditions (Herrera 1995). For instance, the effectiveness of warming via floral or petal movements may vary with floral traits (e.g., size or colour; Shrestha *et al.* 2018; Roddy 2019). In tubular flowers, for instance, those with narrower floral openings may be able to maintain a more distinct internal floral environment than those with wider floral openings (van der Kooi *et al.* 2019). This may be achieved if narrower floral openings provide more resistance to heat transfer between the interior floral environment and the external environment. Environmental variation, such as solar angle and light intensity, should strongly impact the capacity for passive warming via the absorption of solar radiation (Stanton & Galen 1989; Galen 2006; Dietrich & Körner 2014). Likewise, variation in evaporative cooling among individuals should be impacted by petal structure and size, ambient temperature, the intensity of solar radiation, and local water availability (Patiño & Grace 2002; Galen 2006). Linking the capacity of floral warming and/or cooling with phenotypic variation in floral traits and environmental variation is important for understanding their combined effects on floral thermoregulation.

Hexastylis arifolia (Aristolochiaceae) is an evergreen understory herb distributed in the Southeastern United States. The flowers are co-sexual with a fleshy (~2-mm thick) basally flared floral tube comprised of modified sepal tissue and are long-lived (anthesis can exceed 30 days if unpollinated). Blooming from February to May, flowers (Fig. 1) are presented on the forest floor, typically concealed by leaf litter. The floral biology of *H. arifolia* is poorly understood, and the unusual geophilic flowering habit presents an interesting set of thermal constraints and opportunities. Specifically, the location of the flowers should limit their capacity for floral warming by absorption of solar radiation while possibly aiding in warming via insulation. Additionally, floral thermogenesis has been documented in other Aristolochiaceae (González & Pabón-Mora 2015), though to date, it is unknown in *Hexastylis* or closely related *Asarum*. Here, we combined several datasets from experiments in both the field and in controlled conditions on the thermal ecology of *H. arifolia* to address the following questions:

1 What is the thermal optimum and tolerance breadth of *H. arifolia* pollen?
2 Do *H. arifolia* flowers show signatures of thermogenesis?
3 What is the relationship between the temperature of the airspace inside the floral tube and outside of flowers in natural conditions?
4 Is floral morphological variation linked with the temperature differential between the interior and exterior floral environment?

MATERIAL AND METHODS

Study species

Hexastylis arifolia (Aristolochiaceae) is an herbaceous perennial native to the Southeastern United States. Plants produce co-sexual tubular urn-shaped flowers with narrow floral openings (Fig. 1). The floral tube is a synsepalous calyx with stomata on abaxial (Figure S1) and adaxial (J. Heiling, pers. obs.) surfaces. Flowers are presented on the forest floor beneath the leaf litter. In the focal area of this study, upstate South Carolina, *H. arifolia* blooms in the spring (late February to early May). The pollination biology of *H. arifolia* is not well documented, though relatives (genera *Hexastylis* and *Asarum*) are pollinated by fungus gnats (Vogel 1978), and it is posited that flowers in Aristolochiaceae are fungal brood site mimics (Sinn *et al.* 2015). Flowers are thought to be self-compatible, and insect activity within flowers enhances seed production (Otte 1978).

Pollen germination thermal performance curve

In late January, we collected 26 plants from the Clemson Experimental Forest (34.64°, −82.82°) to serve as a source of flowers for the thermal performance curve for pollen germinability. We transplanted them into pots with a soil mix of Fafard and Turface (3:1 v:v), fertilized them with Osmocote (14:14:14, NPK), and covered the surface with sterilized leaf litter. Plants

Fig. 1. (A) *Hexastylis arifolia* flowers, (B) image of flowers showing the floral opening and internal floral trichomes, and (C) the base of a flower with the calyx tube removed to reveal dehisced anthers, pollen, and a bifid stigma.

were maintained in a Percival growth chamber with conditions that mimic winter in upstate South Carolina. As a basis for the environmental parameters used to create an artificial winter for plants to induce flowering, we utilized PRISM climate data (PRISM Climate Group). For 3 weeks, conditions were maintained at 2.2 °C at night and 12.2 °C during the day with 10 h light and 72% RH. Then, for 2 weeks, conditions were changed to 5 °C at night and 16.1 °C during the day, with 11 h of light and 74% RH. Lastly, conditions were adjusted to mimic early spring conditions, with nighttime temperatures set to 9.4 °C and daytime temperatures set to 21.1 °C in conjunction with 12 h of light and 74% RH. Upon initiation of flower buds, individuals were moved to a greenhouse set to 18.3 °C. From these plants, we collected flowers to be used to create a thermal performance curve for *H. arifolia* pollen. Because of the slow flower development in *H. arifolia*, and insufficient flower production in the greenhouse, only 11 of the potted plants in the greenhouse provided flowers for experiments. Therefore, we supplemented with flowers collected from three sites near Clemson University: one in the SC Botanical Gardens (coordinates: 32.67°, −82.22°; N = 64 flowers), and two in the Clemson Experimental Forest (coordinates: 34.64°, −82.82°, N = 62 flowers; and 34.69°, −82.89°, N = 11 flowers). In total we collected 148 flowers, 11 from potted greenhouse plants and 137 from field sites to be used for our thermal performance curve experiment. From a given plant, one or two flowers were collected and used in the experiment.

In the lab, flowers were dissected using a razor blade, and pollen was collected using a dissection probe. Pollen from individual flowers was placed in separate vials containing 20 µl 15% sucrose Brewbaker-Kwack solution (Kearns & Inouye 1993) and then placed into a thermocycler such that each round of samples experienced a distinct temperature. The thermocycler programs were composed of seven different one-step programs in which temperature was constant for 24 h (4–40 °C). Lower increments of thermal exposure were tested (10, 12, and 15 h); however, 24 h yielded the greatest proportion of pollen germination. Only one pollen sample was used from each flower which experienced only one of the seven designated temperature regimes (4, 10, 16, 22, 28, 34, or 40 °C). Between 11 and 26 samples were scored per temperature treatment (mean = 21.14 per treatment). After the temperature treatment, pollen samples were removed from the thermocycler, and germination was inhibited with 5 µl Farmer's Fixative (1 acetic acid: 3 EtOH, v:v). Samples were vortexed, and then 10 µl from each sample was mounted on a glass slide, sealed with a coverslip, and secured with clear fingernail varnish. Pollen germination was scored using a standard light microscope at 10×. The presence of a pollen tube at least as long as the diameter of the pollen grain indicated germination. We scored the presence or absence of pollen germination for an average of 210.8 (±110.8 SD) grains per sample and calculated germination proportion. While the number of grains scored in a given sample was very weakly correlated with germination rate ($r = -0.16$, $P = 0.05$), it was unassociated with temperature treatment ($r = -0.08$, $P = 0.33$).

To generate a thermal performance curve (hereafter, TPC) for pollen germinability, we modelled the proportion of pollen germination as a function of temperature with a nonlinear least squares model using four distinct functions: Kumaraswamy, Gaussian, quadratic and beta functions (minpack.lm; Elzhov *et al.* 2016). We generated AIC values for each curve to determine the best fit. The Gaussian model provided a lower AIC (AIC = −366) than the Kumaraswamy (AIC = −362.2), quadratic (AIC = −352.2), and beta (−348.8) functions. The two best fit models generated nearly identical TPCs. Using model fit parameters from the best fit Gaussian function we obtained the thermal optimum for pollen germination (T_{opt}) and the

50% tolerance breadth (B_{50}), which is the width of the TPC at 50% of the maximum performance value (Sheth & Angert 2014; Huey & Stevenson 2015). Because some flowers were collected on potted greenhouse plants (7.4%), while most were field collected, we evaluated whether the inclusion of greenhouse samples impacted the overall response of germination to temperature prior to generating the TPCs. We tested whether the germination dataset with and without greenhouse samples differed in a quadratic response to temperature. The inclusion of greenhouse samples did not impact the linear (temp × dataset, $F_{1,279} = 0.027$, $P = 0.87$) or nonlinear (temp^2 × dataset, $F_{1,279} = 0.034$, $P = 0.85$) effect of temperature on germination, so greenhouse samples were retained.

Test of thermogenesis

To test whether *H. arifolia* has the capacity for thermogenesis, we used seven of the plants collected from the Clemson Experimental Forest that were maintained in the greenhouse until the initiation of flowers. Once the plants presented buds, they were kept in the lab at room temperature under relatively stable conditions (approx. 15.0–22.4 °C) over the course of the 6 week-long study. As soon as each flower began to open, we inserted a fine-wire K-type thermocouple probe into the opening of a single flower from each potted plant. No damage was imposed on the surrounding floral tissue. This thermocouple probe measured the air space inside of the floral tube experienced by pollen and ovules. Flowers were covered with leaf litter to mimic natural conditions. We adhered a thermocouple probe to a florist stick at flower level but approximately 4 cm outside of the floral opening to track ambient temperature. Thermocouple probes were attached to an Omega datalogger (Omega Engineering., Norwalk, CT, USA) which recorded temperature in 1-min intervals. We tracked internal and ambient floral temperature for a range of 138–862 h per flower. When a flower indicated signs of withering, we removed the internal thermocouple probe to cease temperature recording. We could not record the development of the reproductive stage (e.g., anther dehiscence) for two primary reasons. First, because the floral openings are small, we could not visually inspect the floral interior without damaging the floral tube. Second, removing calyx tissue to record the reproductive stage would have disturbed the thermal microenvironment of the flower.

To define incidences of thermogenesis, we used a cutoff of the floral interior exceeding 1.5 °C or above ambient temperature, following Claudel *et al.* (2023), for at least 4 consecutive minutes. To do this, we calculated the difference between floral interior temperature and ambient temperature (ΔT), recorded the start time, duration (minutes and seconds), and range of warming during each period of thermogenesis for each flower.

Temperature in the field

To evaluate the thermal dynamics of flowers in the field, we tracked external and internal floral temperatures of single flowers on 40 naturally occurring plants in the South Carolina Botanical Gardens between 24 February and 17 April 2022. We removed leaf litter from flowers, placed K-type thermocouple probes inside of the floral tube, and affixed a thermocouple probe to a florist stick at flower level within ~3 cm of the flower to measure external floral temperature. Prior to logging temperature, internal and external probes were re-covered with leaf litter to mimic the previously undisturbed environment. Probes inside of flowers measured the temperature of the air space experienced by pollen and ovules, while external probes measured the temperature experienced by flowers under the leaf litter. It is possible that external probes could have been measuring air space under leaf litter or touching leaf litter itself. However, this is the environment experienced by *Hexastylis* flowers in natural conditions. Probes were attached to an Omega datalogger that tracked the external and internal floral temperature every minute over a 24-h period. On each flower for which we logged temperature, we measured the opening width of the floral tube and floral length using digital callipers.

To address whether internal floral temperature deviated significantly from ambient temperature, and to evaluate diurnal patterns in such deviation, we computed an average interior floral temperature and external ambient temperature for each of six 4-h time bins for each flower following Heiling & Koski (2024) (06:00–09:59, 10:00–14:00, 14:01–18:00, 18:01–22:00, 22:01–02:00, 02:01–06:00 h). These time bins were selected because they capture characteristic temperature shifts throughout the diurnal cycle, with the first (06:00–09:59 h) capturing the initial warming period coincident with the onset of daylight, followed by the periods capturing the daytime highs (10:00–14:00 and 14:01–18:00 h), and the final three (18:01–22:00, 22:01–02:00, 02:01–06:00 h), capturing the continual drop in temperature from sunset through early morning. We modelled temperature as a function of probe location (inside floral tube vs. outside floral tube), time bin, and their interaction, with individual flower identity as a random term using a linear mixed effects model (lme4 R package; Bates *et al.* 2015). Upon finding a significant probe location × time bin interaction, we tested whether the floral interior and ambient temperature were significantly different within each time bin using post-hoc Tukey tests adjusted for multiple comparisons using the emmeans package (emmeans R package; Lenth 2022).

We then assessed whether the deviation of internal floral temperature from ambient temperature was dependent on ambient temperature. We calculated floral ΔT as ambient temperature subtracted from internal floral temperature, where zero indicates flowers that are isothermal with their environment, positive values indicate warmer flowers, and negative values indicate cooler flowers. ΔT was calculated for each time bin for each flower. We visually assessed a scatterplot of ΔT against ambient temperature and observed a nonlinear relationship. We fit two-, three-, and four-order polynomial regressions to the data and determined the best fit by comparing models using the ANOVA function in R. A two-order polynomial regression outperformed a linear regression ($F = 28.6$, $P < 0.0001$), and a three-order outperformed the two-order ($F = 31.5$, $P < 0.0001$) while the four-order polynomial did not outperform the three-order ($F = 3.954$, $P = 0.07$). Thus, we present the three-order polynomial fit. With the three-order polynomial equation, we calculated the ambient temperature at which ΔT crossed 0 to estimate the ambient temperature at which flowers shifted from warmer (ΔT > 0) to cooler (ΔT < 0) than ambient.

To determine whether the capacity for modifying internal floral temperature was associated with floral morphology, we tested whether midday ΔT values (10:00–14:00 h) were

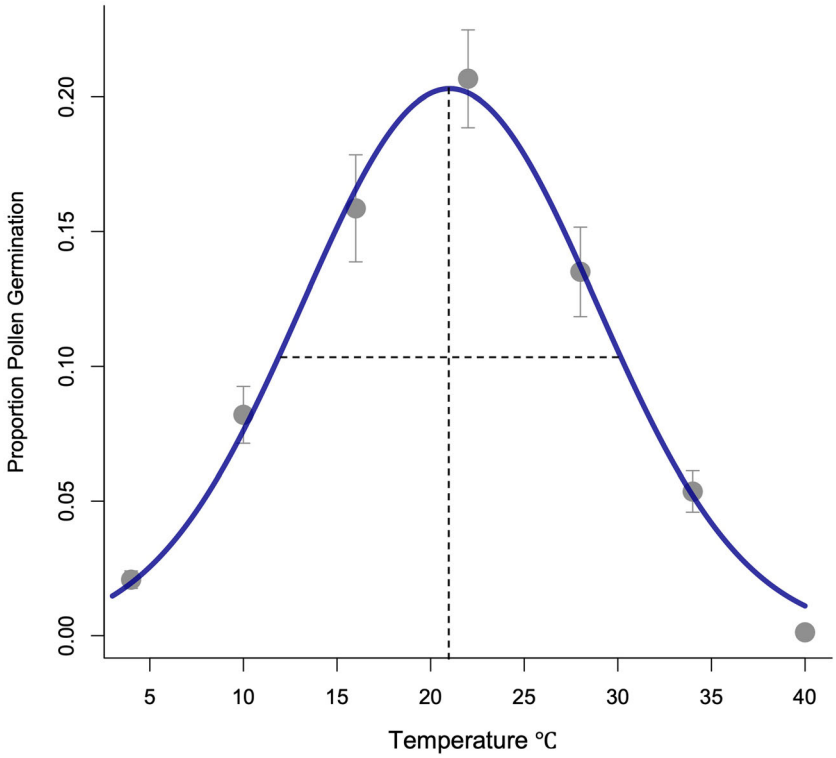

Fig. 2. Thermal performance curve of pollen germination of *Hexastylis arifolia* fit using a Gaussian function. Data points at seen temperature treatments represent mean pollen germination ±1 SE. The vertical dotted line denotes the thermal optimum (T_{opt}), and the horizontal dotted line denotes the 50% tolerance breadth (B_{50}).

associated with the floral opening width. We chose 10:00–14:00 h because this was the only time-period in which interior floral temperature significantly differed from ambient temperature (see results). We predicted that flowers with narrower openings would have more negative ΔT (i.e., be relatively cooler than ambient temperature) than those with larger openings, as they might provide greater thermal resistance to heat flux from the surrounding environment. This inference was made based on our knowledge that the ambient temperature was frequently higher than the temperature inside the flower (see Results). Thus, per the second law of thermodynamics, warmer air from the environment will spontaneously move into cooler areas (i.e., the space within the calyx tube). To test this, we modelled ΔT as a function of floral opening width, ambient temperature, and their interaction. We included floral length as a covariate in the model to account for variation in flower size. We further predicted that flowers with narrower openings should also maintain a more stable thermal environment than those with larger openings due to greater resistance to the movement of air between the internal and external floral environment. We calculated the coefficient of variation (CV) of internal floral temperature from 10:00–14:00 h using the temperature recordings at 1-min intervals. Because this should be strongly influenced by variance in temperature outside of the flower, we also calculated the CV of temperature from each flower's associated external thermocouple probe. We modelled floral temperature CV as a function of the floral opening width, external temperature CV and their interaction with floral tube length as a covariate. For modelling both ΔT and temperature CV, we used standard linear models.

RESULTS

Thermal performance curve for pollen germination

A Gaussian formula predicted the response of pollen germination to temperature (Fig. 2; all parameter estimates, $P < 0.0001$). The performance optimum for pollen germination of 20.3% was at 21.01 °C (±0.508 SE), and the 50% tolerance breadth ranged from 11.76 to 30.28 °C. Pollen did not germinate in the warmest treatment of 40 °C.

Thermogenesis

Six of the seven plants tested for floral thermogenesis exhibited at least one spike in internal floral temperature of ≥1.5 °C for ≥4 min (Table 1). All individuals exhibiting floral thermogenesis initiated warming within a narrow timeframe between 08:04 and 08:53 h, and the duration of warming ranged from 4 to 23 min. The maximum thermogenesis recorded was 4.8 °C above ambient temperature. Most individuals were thermogenic on only a single day (Table 1); however, thermogenesis was recorded on four separate days for one individual (Table 1). Temperature time series plots depicting thermogenic activity on three separate days are provided in Figure S2.

Floral thermal dynamics in the field

Across 40 naturally occurring flowers in the field, average temperature inside the floral tube deviated significantly from external ambient temperature only during the warmest part of the

Table 1. The date, time of initiation, duration, and temperature range of floral thermogenesis (°C above ambient temperature) from seven *Hexastylis arifolia* individuals maintained in a laboratory setting.

		incidence of thermogenesis			
flower ID	total hours recorded	date	start time	duration (min)	ΔT range (°C)
1	406.2	3/27/23	08:31	6	1.6–3.0
2	364.0	3/27/23	08:42	10	1.5–2.4
3	524.3	–	–	–	–
4	862.4	4/18/23	08:04	21	1.5–4.8
5	862.4	4/15/23	08:22	23	1.5–2.9
6	138.4	3/14/23	08:44	13	2.2–3.9
		3/15/23	08:53	4	1.6–2.1
		3/16/23	08:52	6	1.6–2.4
		3/19/23	08:42	15	1.8–3.6
7	862.4	4/12/23	08:49	5	1.5–2.2

We considered any record of an internal floral temperature >1.5 °C above ambient temperature for 4+ min as an incidence of thermogenesis. ΔT is the temperature inside the floral tube minus air temperature outside of the floral tube.

day between 10:00 and 14:00 h (probe location x time bin, $X^2 = 20.95$, $P < 0.001$; Fig. 3). During this period, flowers fell 1.87 °C below flower-level ambient temperature, on average (flower = 18.78 °C, ambient = 20.65 °C, $t = 3.96$, $P = 0.005$). The range in ΔT across individual flowers during this time period was, however, wide, ranging from −9.9 to +3.0 °C, and only five of 40 individual flowers had positive ΔT values.

The ΔT was predicted by ambient temperature ($R^2 = 0.55$; Fig. 4). When ambient temperature was cooler, the internal floral environment was warmer than ambient, but when ambient temperatures were higher, internal flower temperature was cooler than ambient. The predicted ambient temperature at which flowers became warmer or cooler than ambient (i.e., y = 0 in Fig. 4) was 11.4 °C. This is very close to the lower bound of the 50% tolerance breadth for pollen germination (11.76 °C; Fig. 4). Moreover, ΔT was most negative at ambient temperatures near the upper limit of the 50% tolerance breadth of pollen (Fig. 4).

Variation in floral opening width among flowers mediated ΔT during peak heat (10:00–14:00 h; Fig. 5A). Specifically, the interior of flowers with narrower openings fell further below ambient temperature than those with wider openings when it was warm (Fig. 5A; Opening Width × Ambient Temp. $F_{1,35} = 10.76$; $P = 0.002$). Floral length was a significant covariate in the model predicting ΔT, with shorter flowers having lower ΔT regardless of ambient temperature (Length $F_{1,35} = 10.41$, $P = 0.002$). Floral opening width also mediated the stability of internal floral temperature during peak heat. Specifically, the interiors of flowers with narrower openings were more thermally stable than flowers with wider openings (Fig. 5B; Opening width $F_{1,35} = 6.17$; $P = 0.018$). While temperature variance within flowers was strongly positively associated with thermal variation outside of the flower (Ambient Temp. CV $F_{1,35} = 10.62$, $P = 0.002$), there was no interaction between opening width and ambient temperature CV ($P = 0.19$), and there was no effect of floral tube length on thermal variation inside of the flower ($P = 0.42$).

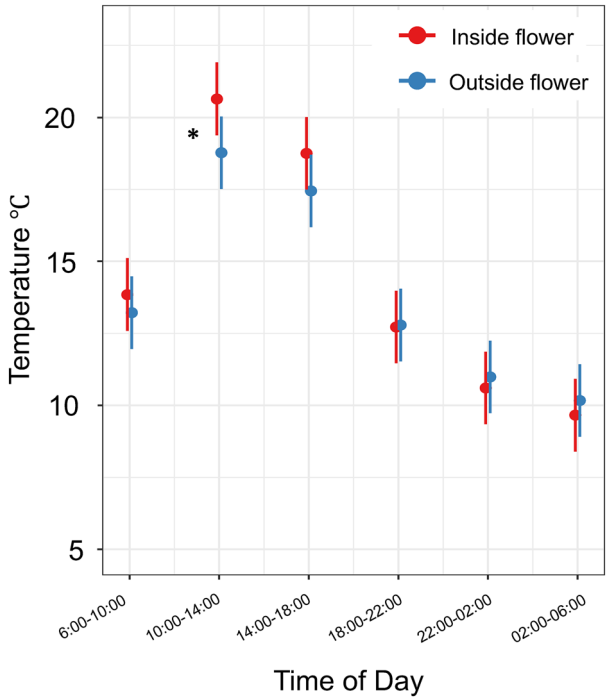

Fig. 3. Average temperature of the floral environment (the air space inside of the floral tube) and ambient temperature adjacent to flowers (under leaf litter outside of the floral tube) from 40 *Hexastylis arifolia* flowers in natural conditions across a 24-h time-period. Flowers were significantly cooler than ambient temperature only during peak daytime heat (10:00–14:00 h).

DISCUSSION

Flowering plants are often subject to strong diurnal and seasonal variation in temperature. Because pollen and ovules are temperature sensitive, the temperature of the pollen and ovule environment within flowers can greatly impact plant fitness. Therefore, there are likely reproductive benefits to floral thermoregulation. In controlled conditions, *H. arifolia* flowers exhibited short periods of thermogenic activity, usually on a single day during the floral lifespan, that may be associated with floral developmental change, such as anther dehiscence. In field conditions, the temperature of the interior floral environment reached up to 4 °C higher than temperatures outside of the flower under cool ambient conditions, but in some cases fell nearly 10 °C below external temperatures under warmer ambient conditions. When ambient temperatures fell below the lower tolerance breadth of pollen viability, flowers tended to warm or retain heat such that internal floral temperatures remained within the tolerance zone for pollen germination (Fig. 4). Likewise, observed floral cooling at extreme high temperatures should maintain a floral environment nearer the optimum for pollen. Under scenarios of rapid climate warming, traits that afford the capacity for floral cooling may become increasingly important for plant reproductive fitness.

Floral thermogenesis in *H. arifolia* was very short-lived relative to the entire lifespan of a flower, ranging only 4–23 min. The sexual stage of flowers is not readily observable in the urn-shaped flowers of *H. arifolia*, and floral dissections to record reproductive development would have altered the temperature of the interior floral environment, obscuring our

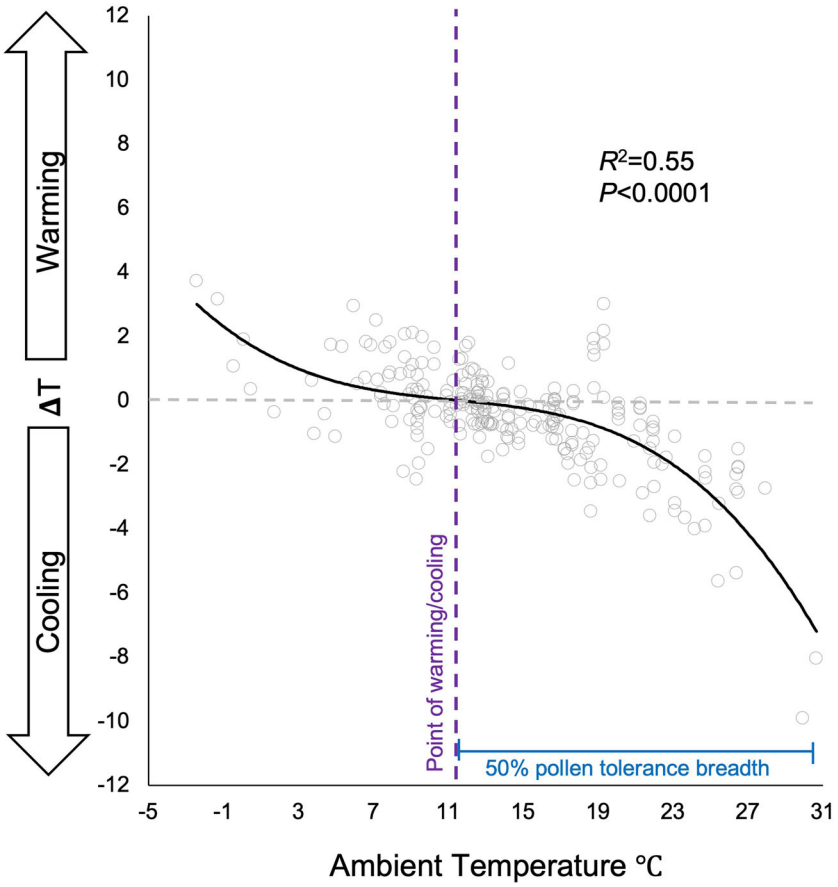

Fig. 4. The differential between temperature inside of the floral tube and ambient temperature (ΔT) plotted against ambient temperature for 40 *Hexastylis arifolia* flowers in natural conditions. Each point represents a 4-h average ΔT for a single flower. A three-order polynomial regression fit better than a two-order or four-order polynomial. The estimated ambient temperature at which ΔT = 0 is shown with a purple dashed line, and the 50% tolerance breadth for pollen germinability is depicted along the *x*-axis in blue.

ability to detect thermogenesis. Despite this, the short duration and conserved timing of thermogenesis across flowers on separate plants suggest that warming may be associated with a transition in floral development. Cellular separation processes regulated by metabolic reactions (Ranjan *et al.* 2017) have the potential to result in thermogenesis as a byproduct of metabolism. Floral thermogenesis has been linked to specific sexual phases in other taxa (Wang *et al.* 2013; Claudel *et al.* 2023). However, sex stage-specific thermogenesis in other taxa often lasts for multiple days over which a given sexual stage persists in the flower or inflorescence (Claudel *et al.* 2023). We suggest that thermogenesis in *H. arifolia* is unlikely to aid in increased pollinator attraction through a direct heat reward or by increased emission of volatiles. Instead, thermogenesis is likely caused by heat release from a cellular developmental process.

In the field, the interior air temperature of *H. arifolia* flowers was cooler than external temperature during the hottest times of the day, but warmer during cool ambient temperatures. The cooling effect of the floral interior was stronger than warming – internal floral temperatures fell more than 9 °C below external floral temperature in some cases, while positive temperature differentials only reached ~4 °C (Fig. 4). A shift from flowers being cooler to flowers being warmer than ambient conditions was predicted when ambient temperatures were near 11.64 °C. Given that the lower bound of the 50% tolerance breadth for pollen germination was 11.7 °C, positive ΔT at cool temperatures should increase the viability of pollen. Likewise, negative ΔT at ambient temperatures above the optimum for pollen germination (~21 °C) should bring floral interiors closer to the thermal optimum for pollen. Together, our data suggest that floral thermoregulation has the potential to enhance pollen performance under unfavourable ambient thermal conditions.

We suggest evapotranspiration could be a primary mechanism driving negative ΔT values under higher ambient temperatures for several reasons. First, the fleshy floral tube is comprised of thick modified sepal tissue (Fig. 1), which likely can retain substantial amounts of water. Sepal tissue has been shown to be important for evaporative floral cooling in other taxa (Patiño & Grace 2002) and floral structures have been shown to be leakier than vegetative structures (Roddy *et al.* 2023), especially during drought (Bourbia *et al.* 2020). Second, the abaxial surface of the calyx tube has stomata (Figure S1) so the floral tissue is capable of gas exchange. Finally, the presence of trichomes within the flower may slow the loss of moist air to the external floral environment (Lusa *et al.* 2014). Furthermore, flowers with narrower openings were more thermally stable (Fig. 5B) and cooler than ambient

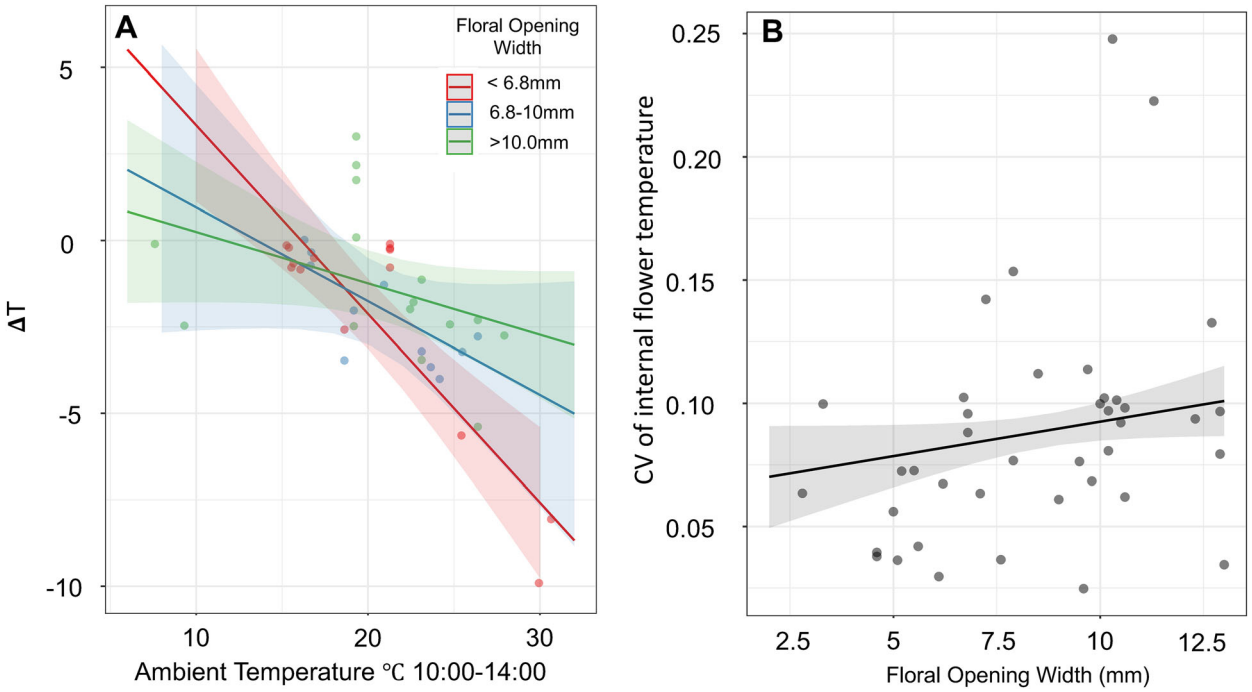

Fig. 5. (A) Floral opening width predicted the relationship between ΔT and ambient temperature for *Hexastylis arifolia* flowers in natural conditions during 10:00–14:00 h. Flowers with narrower openings had more strongly negative ΔT than those with wider openings when external floral temperatures were warm (Opening Width × Ambient Temp. $P = 0.002$). Each point represents a 4-h average ΔT for a flower. Flowers are colour-coded by floral opening width. (B) Flowers with narrower openings experienced less variation in internal flower temperature (coefficient of variation, CV) from 10:00 to 14:00 h than those with wider openings ($P = 0.002$).

temperatures when it was hot (Fig. 5A). Thus, a narrower floral opening may aid in maintaining a distinct internal floral microenvironment by limiting heat exchange between the exterior ambient air and the floral interior (Davletshin & Mikheev 2012). We note, however, that the extreme cooling events observed (8 °C or more below ambient) were rare (Fig. 4) and thus may only occur under very specific environmental and physiological conditions (e.g., very low relative humidity, and low stomatal resistance). Thus, more data would be required to determine how frequent are such cooling events, and experimental approaches (e.g., Patiño & Grace 2002) are required to confirm the role of evaporative cooling.

Should selection act on floral temperature in *H. arifolia*, traits that afford enhanced cooling may become increasingly important for reproductive fitness under warmer conditions and an increased frequency of extreme heat events. For instance, *H. arifolia* flowers with smaller floral openings may be favoured by selection because of stronger resistance to high external floral temperatures that may damage pollen. Under a high emissions scenario, Pickens County, SC (location of our field study) is projected to experience 50 spring days with maximum temperatures exceeding 35 °C, while the historical average days with maximum temperatures exceeding 35 °C is 5.07 days (1961–1990; U.S. Federal Government 2023, NOAA Climate Explorer). Therefore, internal floral cooling, which we showed is stronger under higher environmental temperatures, should become increasingly favourable for *H. arifolia* pollen fitness in the focal region of this study, especially considering our laboratory study showing that pollen failed to germinate at 40 °C (Fig. 2).

The instances of positive ΔT values observed in the field (Fig. 4) are unlikely driven by the modest and brief thermogenesis measured in laboratory conditions (Figure S2). While drivers of floral warming in field conditions are unclear, it is possible that instances of warmer internal floral environments relative to external conditions may be driven through the retention of heat by floral tissue or within the airspace inside flowers. For instance, the fleshy calyx tube may have a high water content and may cool more slowly than air temperatures due to the high specific heat of water and latent heat retention. Pubescence near the floral aperture (Fig. 1B) could also slow the transfer of heat from the floral interior to the floral exterior, as pubescence has been implicated in floral thermal dynamics in other systems (van der Kooi *et al.* 2019).

In addition to direct impacts on reproductive fitness through modifying gamete viability, floral temperature can impact pollinator interactions with flowers (Dyer *et al.* 2006; van der Kooi *et al.* 2019). The pollination biology of *Hexastylis* spp. has not been well studied, but closely related species are pollinated by fungus gnats and other dipterans (Sinn *et al.* 2015). It is thought that the flowers of *H. arifolia* act as brood site mimics for fungus gnats (order Diptera) because the location of the flowers (beneath leaf litter), and the microenvironment within the "jug" of the floral tube (shaded, cool, and moist) are in line with fly pollination. Additionally, minute eggs have been observed within field-collected flowers upon dissection (Heiling, pers. obs.). The role of fungus gnats as pollinators in *H. arifolia* has yet to be well documented, but some speculate that some flowers are dependent on mimesis and the attraction of these micro-dipterans (Sinn *et al.* 2015; Han *et al.* 2022). Moreover, the cooler internal floral temperatures observed during periods of high ambient temperature could serve as a thermal reward for fungus gnats, as they are most sensitive to

warmer temperatures during the winter and spring (Fitzgerald *et al.* 2021). In addition to narrow floral openings, trichomes lining the inside of the flower may trap the humid air, making it an ideal location for desiccation-sensitive dipteran larvae (Barbosa *et al.* 2021). A detailed profile of the pollination ecology of *H. arifolia* is currently under investigation.

In summary, we combined controlled experiments and field data to link the thermal biology of *H. arifolia* flowers with morphological variation and with the thermal performance of pollen. While the pollination ecology of *H. arifolia* remains to be investigated, it is unlikely that the brief and modest instances of thermogenesis detected under laboratory conditions enhance pollination success. Instead, flowers with interiors that are cooler than exterior ambient temperatures observed under especially warm field conditions can potentially maintain an internal floral environment suitable for pollen performance. Variation in the degree of floral cooling and the stability of internal floral temperature among individuals was mediated by floral opening width. Because temperature directly impacts gametophyte performance, floral thermoregulatory capacity and the floral traits associated with it could experience phenotypic selection.

AUTHOR CONTRIBUTIONS

TNS conducted experiments and collected data in the field, curated data, analyzed data and wrote the first draft of the manuscript. JMH managed data collection, collected data, curated data and provided feedback on manuscript drafts. MHK obtained funding, managed the project, analyzed data, made figures and provided feedback on manuscript drafts.

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DATA AVAILABILITY STATEMENT

All data and code associated with this manuscript are available here: https://zenodo.org/records/10631572.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. An epidermal peel from the abaxial (outer) surface of the synsepalous calyx, with stomata labelled with blue boxes.

Figure S2. Time series of floral and ambient temperature, showing instances of thermogenesis in *Hexastylis arifolia* flowers on three separate days.

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