Flower orientation influences floral temperature, pollinator visits, and plant fitness

Nicky M. Creux[1,4], Evan A. Brown[2], Austin G. Garner[2], Sana Saeed[1], C. Lane Scher[2], Srinidhi V. Holalu[3], Daniel Yang[3], Julin N. Maloof[1], Benjamin K. Blackman[2,3] and Stacey L. Harmer[1]*

Author Addresses

[1]Department of Plant Biology, University of California, One Shields Avenue, Davis, CA 95616, USA

[2]Department of Biology, University of Virginia, PO Box 400328, Charlottesville, VA 22904, USA.

[3]Department of Plant and Microbial Biology, University of California, 111 Koshland Hall, Berkeley, CA 94720, USA.

[4]Department of Plant and Soil Sciences, FABI, Innovation Africa, University of Pretoria, Lynwood road, Hatfield 0002, South Africa

ORCID identifiers:
Nicky M. Creux, 0000-0002-4179-6995; Evan A. Brown, 0000-0002-1398-5721; Austin G. Garner, 0000-0001-8279-8260; C. Lane Scher, 0000-0003-3689-5769; Srinidhi V. Holalu, 0000-0002-1948-8216; Daniel Yang, 0000-0002-5614-6055; Julin N. Maloof, 0000-0002-9623-2599; Benjamin K. Blackman, 0000-0003-4936-6153; and Stacey L. Harmer, 0000-0001-6813-6682

Corresponding Author:
*Prof Stacey Harmer
Department of Plant Biology, University of California,
One Shields Avenue, Davis, CA 95616, USA
+1 530 752 8101
slharmer@ucdavis.edu

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Social media accounts:

Nicky M. Creux, twitter: @NickyCreux; Evan A. Brown, twitter: @evanbbiosci; C. Lane Scher, twitter: @lane_scher,; Julin N. Maloof, twitter: @jnmaloof; Benjamin K. Blackman, twitter lab hashtag #blackmanlab, facebook username: bkblackman

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Summary

- Effective insect pollination requires appropriate responses to internal and external environmental cues in both the plant and the pollinator. *Helianthus annuus*, a highly outcrossing species, is remarkable for its uniform eastward orientation of mature pseudanthia, or capitula. Here we investigate how this orientation affects floral microclimate and the consequent effects on plant and pollinator interactions and reproductive fitness.
- We artificially manipulated sunflower capitulum orientation and temperature in both field and controlled conditions and assessed flower physiology, pollinator visits, seed traits, and siring success.
- East-facing capitula were found to have earlier style elongation, pollen presentation, and pollinator visits compared to capitula manipulated to face west. East-facing capitula also sired more offspring than oppositely oriented capitula and in some conditions produced heavier and better-filled seeds. Local ambient temperature change on the capitulum was found to be a key factor regulating the timing of style elongation, pollen emergence, and pollinator visits.
- These results indicate that eastward capitulum orientation helps control daily rhythms in floral temperature, with direct consequences on the timing of style elongation and pollen emergence, pollinator visitation, and plant fitness.

Introduction

Effective cross-pollination requires daily and seasonal coordination between plants and their pollinators. To facilitate these interactions, plants have evolved a number of important floral traits to attract pollinators and ensure pollen transfer and fertilization, including flower and floral organ shape, size, color, orientation, markings, scent, and movement (Kevan 1975; Rosas-Guerrero *et al.*, 2014; Haverkamp *et al.*, 2019). Correspondingly, pollinators also display a range of behavioural adaptations that facilitate efficient foraging on different plant species (Macior, 1974). In many cases, the expression of both floral traits and pollinator behavioural adaptations are regulated by the organisms' internal circadian clocks (Moore, 2001; van Doorn & Kamdee, 2014; Bloch *et al.*, 2017). Circadian clocks are important integrators of external environmental cues, such as light or temperature, enabling organisms to receive and respond to these cues at the appropriate time of day (Tomioka & Matsumoto, 2009; Creux & Harmer, 2019). Recent studies have found that plant and pollinator clocks not only regulate floral or behavioural traits in their respective organisms, but also interact to coordinate the precise timing of pollination (Yon *et al.*, 2017a; Yon *et al.*, 2017b; Fenske *et al.*, 2018). These studies suggest that successful insect-mediated pollination involves intricate signalling between the circadian clock and environmental cues to maintain the timing within each organism as well as between organisms to ensure plant reproductive success.

Floral temperature is another important factor that regulates and coordinates plant-pollinator interactions, either by directly promoting or modifying pollinator foraging behaviour or by indirectly amplifying floral reward signals on the flowers to attract pollinators (Heinrich 1972; Sagae *et al.*, 2014; Harrap *et al.*, 2017). For example, some pollinators such as bumble bees require less energy to reach the appropriate flight temperature when visiting warmer flowers (Sapir *et al.*, 2005; Rands & Whitney, 2008). Another study has suggested that pollinators might change feeding behaviour based on flower temperature, selecting cooler plants in high temperatures and warmer plants during cool periods (Norgate *et al.*, 2010). In addition to influencing pollinator visitation, floral temperature also exerts substantial influence on development and viability of germ cells and seeds. Consequently, many plant species have evolved adaptations that manipulate the floral microclimate to maintain physiologically permissive or optimal temperatures (Corbet, 1990; van der Kooi *et al.*, 2019). Well-known examples include the thick petals of magnolias that keep the core floral temperature raised in these early spring blooms (Wang *et al.*, 2013); plasticity of flower pigmentation in *Plantago* species to modulate temperature of the flowers through the season (Lacey & Herr, 2005); and solar tracking by the peduncles of alpine buttercups, which maintains core floral temperatures during seed development (Stanton & Galen, 1989).

Ample literature has investigated how heat stress negatively affects plant fitness through impaired pollen donation (also referred to as male fitness), including inhibition of pollen development, pollen emergence and fertility (Hedhly, 2011; Giorno *et al.*, 2013; Paupière *et al.*, 2014; Mayer *et al.*, 2015; Dwivedi *et al.*, 2017; Begcy *et al.*, 2019; Raja *et al.*, 2019). Negative effects of heat stress on fitness through fruit and seed production (also referred to as female fitness) have been less well explored, but emerging studies on cereal pistils have shown that the accumulation of reactive oxygen species under high temperatures can reduce stigma receptivity and pollination (Jagadish, 2020). High temperature stress can also affect the timing and development of stamens, in turn altering the synchronicity between stamen and pistil elongation and leading to a shift from selfing to out-crossing in some species (Sakata *et al.*, 2010; Bishop *et al.*, 2016; Pan *et al.*, 2017; Pan *et al.*, 2018). In addition to this temperature-dependent mating system plasticity, genetically-based changes in floral organ development that facilitate adaptive shifts from selfing to out-crossing have been documented in both *Solanum* and *Asteraceae* species (Motten & Stone, 2000; Chen *et al.*, 2007; Vosters *et al.*, 2014; Irwin *et al.*, 2016; Love *et al.*, 2016). Most studies to date have investigated the effects of high heat stress on anthesis and pollination. However, the effects of daily fluctuations within a standard rather than stressful temperature range on the precise timing of developmental events during pollination have received far less attention.

The *Asteraceae* is one of the largest plant families, and includes a number of economically important species such as sunflower, lettuce, and safflower, which are all characterised by a distinctive, compressed, complex inflorescence called the capitulum (Funk *et al.*, 2009). Domesticated sunflower (*Helianthus annuus* L.) provides an excellent *Asteraceae* model for studying the process of anthesis due to the ample genomic resources available and because their large capitula contain thousands of individual florets that undergo anthesis over several days (Putt, 1940; Stuessy *et al.*, 1986; Sun & Ganders, 1990; Andersson, 2008; Badouin *et al.*, 2017; Terzić *et al.*, 2017). A major adaptation for pollinator attraction in sunflowers is the development of ray florets, the elongated, flattened corollas of the outer sterile whorl that are brightly colored and often have UV nectar guides (Wojtaszek & Maier, 2014; Terzić *et al.*, 2017, Todesco *et al.*, 2021). The inner disk florets are fertile flowers, which are developmentally distinct from one another. The florets located towards the outer surface of the capitulum are the first to mature, while the florets in the center are the last to develop (Fig. 1).

Many *Asteraceae* flowers, including sunflowers, are protandrous, terminal stylar presenters with active pollen placement (Howell *et al.*, 1993). The perfect flowers first proceed through a staminate phase, where the male reproductive organs (stamens) reach maturity, before entering a pistillate phase, in which the female organs (pistils) attain maturity (Fig. 1). After the corolla opens at dawn, the anther filaments and style begin to elongate so that the anther tube, formed by the five fused anthers, can emerge, and pollen is released inside the tube. The style elongates more slowly than the anther filaments, and as it does so, it pushes through the center of the anther tube, thereby actively extruding pollen. Only later do the semi-dry stigmas fully emerge and become receptive to pollen (Putt, 1940; Lobello *et al.*, 2000; Sharma & Bhatla, 2013). In this way, male and female reproductive organs mature in close proximity to each other while the difference in elongation timing still thwarts self-pollination of a single floret.

Sunflowers are well known for the near-uniform eastward orientation of mature capitula, an adaptation that we have previously shown affects floral temperature and pollinator visitation (Atamian *et al.*, 2016). In this study, we investigate the daily dynamics of east-oriented sunflower capitula and experimentally re-oriented west-facing sunflower capitula at anthesis and measure the developmental and ecological impacts of capitulum orientation. We describe how environmental cues lead to the proper timing of floral developmental events, which promote cross-pollination and reproductive success. Unlike most previous studies that have conducted end-point analyses of floral traits, we assess the kinetics of plant development in natural and controlled environments. By taking detailed physiological measurements and counting insect visits over time, we found that capitulum orientation affects seed filling in a locality-specific manner with east-facing plants producing heavier seeds. We also found that east-facing capitula confer a male fitness advantage to these flowers, allowing them to sire more offspring than west-facing capitula possibly due to temperature-dependent changes in the timing of anthesis. Our studies on time-of-day specific interactions between plants, pollinators, and the environment suggest that environmental and circadian regulation of capitulum orientation in sunflower (Atamian *et al.*, 2016) controls the floret microclimate to enhance pollinator visits and promote plant fitness.

Materials and Methods

A summary of all experiments and measurements performed is provided in Supporting Information Table S1.

Plant material and growth conditions

The *Helianthus annuus* cultivar HA 412HO (Germplasm Resources Information Network ID: PI 603993; https://npgsweb.ars-grin.gov) was used for all experiments unless otherwise noted. See Supporting Information Methods S1 for details on general growth conditions and Supporting Information Methods S2 for details on the siring experiments.

Field manipulations and data collection

Field plants were monitored for the cessation of heliotropism just before the onset of anthesis, and at this time every second plant in the row was rotated 180° to face the opposite direction (west), while the other plants were kept facing east. Capitulum temperatures and ambient air temperatures were continuously monitored in the field using K-type thermocouples and four channel dataloggers (OMEGA, Norwalk, CT, USA), where the thermocouple was inserted into the center of each capitulum or remained coiled in plastic container housing the logger for the ambient temperature. Pollinator counts were made from 20 min videos that were taken at 30 min intervals from 8:00 am to 9:30 am. One plant per treatment (east, west or west heated) per day for 9-10 days was filmed with Bloggie video cameras (SONY, Tokyo, Japan) on standard tripods. Videos were manually scanned and insects landing on the flower head counted. Some west-facing capitula were heated in the field using an electric dish heater with heat flow directed at the capitulum (H-500, Optimus Enterprise Inc., Anaheim, CA, USA). The distance of the heater from the capitulum was continually adjusted to ensure the temperature matched the corresponding temperatures observed on east-facing capitulum. Whole florets or styles were imaged in the field with a Nikon COOLPIX A (Nikon, Tokyo, Japan) camera. Images of east- and west-facing capitula were also acquired with the COOLPIX A camera on the macro setting, every 15 min from 7:00 am to 9:30 am. Images were manually inspected for timing of pollen extrusion; all anthers with visible pollen in each photograph were counted using ImageJ (Schneider *et al.*, 2012). All time points were adjusted to ZT time with first light as ZT0. A two-way ANOVA with multiple comparisons for factors time and capitulum orientation ($P < 0.05$) was used to determine differences between east-facing, west-facing and west-facing heated plants within each time point. Full-spectrum (400 – 700 nm) and UV-A-only (350 – 400 nm) images were taken of east-facing and west-facing capitula at ZT3 using a Canon DSLR camera or an identical camera modified with a UV band pass filter (LifePixel, Mukilteo, WA, USA). Insect visitations to east- and west-facing disks of wild *H. annuus* accessions from Oklahoma and Texas were observed from late September through October 2019 at Davis, California (CA). Flowers were secured with a string to a wooden post to face cardinal east or west, and insect visitations were captured at 5 min intervals using a Wingscapes Birdcam Pro time-lapse cameras (Moultrie Inc). Cameras were shifted to film newly open flowers after anthesis of all florets in flowers under observation (~3 d). Insect visitation counts and time-stamps were recorded from the pictures and converted to insect visitations per hour relative to the daily time of sunrise. The data was collected over 18-20 days of observation from 2-3 flowers per plant facing either direction for 6-7 plants per population. For each accession, visitation count data between ZT1 and ZT2 was analysed with generalized linear mixed models including day of observation as a random effect and direction as fixed effect with Poisson distribution and log link in R-package *glmmTMB*.

Floret dissection in field and chamber conditions

Individual florets were removed from capitula with forceps and placed on a white background, alongside a standard ruler for imaging with a Nikon COOLPIX to obtain measurements with ImageJ (Schneider *et al.*, 2012) of the whole floret with emerging anther tube as a proxy for anther filament elongation. Florets were then slit open with sharp nose forceps; the base of the style was grasped through the slit and slid out of the bottom of the floret. Styles were placed on a white background and imaged using the Nikon COOLPIX A macro function. Florets were harvested every 15 min from the start to end of anther filament elongation and 3-5 florets were measured per plant, per treatment, and per time point. Lengths of styles and anthers were measured using ImageJ (Schneider *et al.*, 2012). Two-way ANOVA for time and temperature factors was performed and Loess functions were fit to the data and 95% confidence intervals were determined. Bayesian modeling was used to compare anther and style growth in response to different treatments as described in Supporting Information Methods S3.

Seed traits

In Davis, the disk diameters of ten east- and ten west-facing capitula were measured from plants grown from May - July or July - September of 2016. One hundred seeds were randomly selected from each capitulum and weighed. Sixty seeds were dehulled using a scalpel blade and kernel width was measured using a caliper (Mitutoyo). Comparable data was obtained for two experiments in Charlottesville; the anthesis to harvest period of these experiments ran from mid-July through August 2014 (n = 6-8 plants per orientation per experiment). All seeds harvested per plant were counted and weighed in bulk to obtain average seed mass. Kernel widths were measured and then averaged for 15 seeds per plant. Linear models with trial and year as random effects and direction as fixed effect used to determine effect for all three seed traits.

Results

Sunflower capitulum orientation enhances seed quality in a location-specific manner

In our earlier study, we reported that east-facing capitula are significantly warmer than west-facing capitula in the early hours of the day and that this increased temperature coincided with increased pollinator visits during these hours (Atamian *et al.*, 2016). To assess whether capitulum orientation affects multiple aspects of plant fitness, sunflowers were grown in pots in a field setting both in the Mediterranean-type climate of Davis, CA and the moderate-wet climate of Charlottesville, VA (Supporting Information Fig. S1a and S1b). Shortly before opening of the involucral bracts, pots were positioned so that capitula faced east or west throughout anthesis and were left in that position until harvest. At the onset of anthesis, most of the larger leaves have ceased tracking and are aligned roughly parallel to the ground; however a few of the younger leaves closest to the head may continue to track the sun each day until leaf expansion is complete (Shell & Lang, 1976; Lang & Begg, 1979).

At physiological maturity, we measured capitulum diameter and several seed traits. We observed no difference in seed number produced by east- or west-facing capitula in either location (Supporting Information Fig. S2a and S2b). We did observe a significant difference in seed number between the Davis trials planted early in the season and those planted late in the season, with early plantings producing almost twice as many seeds (Supporting Information Fig. S2c). The late Davis planting produced seed numbers comparable to those produced in the early summer in Charlottesville (Supporting Information Fig. S2b and c). This may be due to differences in overall light intensity; in Davis, the peak solar irradiance levels in May – July are higher than those observed in the late summer (August/September) and higher than the irradiance levels observed over the bulk of the Charlottesville growing season (Supporting Information Fig. S1c). These results show that seed number is affected by location and planting time in the season but not by final capitulum orientation (Supporting Information Fig. S2).

We next examined possible effects of orientation on capitulum and seed traits. In the Davis trials, the east-facing capitula were on average 2 cm larger in diameter than their west-facing counterparts (Fig. 2a). We found the difference in capitulum diameter was primarily due to seed size rather than seed number, as dehulled seeds harvested from east-facing capitula were each 0.5 mm wider on average than seeds harvested from west-facing capitula (Fig. 2b). This finding of increased seed size was supported by weight measurements, with seeds from east-facing capitula on average 20% heavier than those from west-facing capitula (Fig. 2c). In contrast, we observed no significant differences in seed weight or seed width between seeds harvested from east- and west-facing capitula grown in Charlottesville (Supporting Information Fig. S3). Thus, capitulum orientation has environment-dependent effects on seed filling but does not affect seed number.

Capitulum orientation affects male fitness by altering siring success

We previously observed that floral orientation impacts pollinator visitation (Atamian *et al.*, 2016) and thus might also impact pollen transfer. Therefore, we next explored whether capitulum orientation influences male fitness by testing the relative siring success of plants with different orientations. Specifically, we surrounded cytoplasmic male sterile (CMS) plants, that require receipt of pollen from other plants to set seed, with genotypically-distinguishable male fertile plants as east- or west-facing sires (Fig. 3a). In five of the seven trials performed, the east-facing genotype sired significantly more offspring on the CMS plants than the west-facing genotype (Fig. 3b). A sixth trial, while not significant, also displayed a similar trend with more offspring sired by the east-facing genotype. Statistical analysis of the seven trials applying repeated G-tests of goodness of fit affirmed that capitulum orientation significantly impacted siring success (Supporting Information Table S2). These findings suggest that capitulum orientation significantly affects plant fitness through pollen transfer, as east-facing sires more successfully competed for ovules compared to west-facing sires.

Capitulum orientation coordinates timing of pollen emergence and pollinator visits

To further understand how capitulum orientation influences siring success, we performed detailed time series analyses of the timing of pollinator visits in the field at Davis, CA. Counts of pollinator visits to east-facing and west-facing capitula during a 20-minute period starting thirty minutes after dawn (Zeitgeber Time (ZT) 0.5) revealed that pollinator visits were significantly higher to east-facing compared to west-facing capitula (Fig. 3c). We observed that insect visits to east-facing but not west-facing capitula increased greatly during the next time window, with east-facing capitula receiving more than 200 visits over the 20-minute period commencing at ZT 1.0 (the highest number of visits across all times) and the number of visits to west-facing capitula only increasing modestly (Fig. 3c). Similarly, we also noted an increase of insect visits on easterly-orientated capitula of wild sunflower populations at ZT 1 (Supporting Information Fig. S4a). Interestingly, from ZT 1.5 onwards, no statistically significant differences in pollinator visits to oppositely oriented capitula were detected (Fig. 3c). Thus, the preference of pollinators for east-facing compared to west-facing capitula is restricted to a relatively short period of time in the early morning.

Since sunflower florets release pollen in the early morning (Putt, 1940), we examined the timing of pollen presentation by east- and west-facing capitula. On east-facing capitula, pollen can first be observed on a small number of florets at ZT 1.25. The initiation of pollen presentation on west-facing capitula is delayed by about 45 minutes, with pollen first observed on a small number of florets at ZT 2.0 (Fig. 4b). The fraction of florets displaying pollen increases steadily on both types of capitula thereafter, with almost all east-facing florets releasing pollen by ZT 2.5 and almost all west-facing florets releasing pollen 30 minutes later, at ZT 3.0. Statistically significant differences between the fractions of florets presenting pollen on east- and west-facing capitula are observed from ZT 1.75 thorough ZT 2.75. Intriguingly, the time delay in pollen release on west-facing capitula (approximately 30 – 45 minutes) correlates with the approximately 30-minute delay between the peak times of insect visits to the two types of capitula (Fig. 3c). A similar delay in the timing of pollen presentation was also observed on differently-oriented wild accessions from Oklahoma and Texas (Supporting Information Fig. S4b-S4c). These data suggest that an orientation-dependent change in the timing of floral development may be a cue leading to earlier pollinator visits.

Capitulum temperature affects the timing of pollen emergence and pollinator visits

We previously demonstrated that floral temperature plays a role in pollinator visits (Atamian *et al.*, 2016). We therefore carried out detailed time course studies examining the effects of temperature on the timing of floral development and insect visits. We found that east- and west-facing capitula are different temperatures through the day, with the fronts of east-facing capitula having higher early morning temperatures and cooler afternoon temperatures than west-facing capitula (Fig. 3d and Supporting Information Fig. S5a-S5d). To isolate temperature from other effects of orientation on floral physiology and insect visits, we artificially heated west-facing capitula in the field to match the temperature of east-facing capitula using the same methods presented in Atamian *et al.*, (2016). We first examined the effects of supplemental heating of west-facing capitula on pollinator visits. During the earliest time window examined (ZT 0.5 to 0.8), supplemental heating had no effect on the numbers of insect visits to west-facing capitula (Fig. 3c). Since we did not observe pollen presentation on any of the three types of capitula before ZT 1.0 (Fig. 4b), we conclude insect preference for east- compared to west-facing capitula between ZT 0.5 and 0.8 is neither due to differences in temperature nor pollen rewards but may be due to other environmental factors such as incident light (van der Kooi, 2016).

Slightly later in the morning, between ZT 1.0 to 1.3, we found that insect visits to west-facing, heated capitula but not unheated west-facing capitula increased considerably compared to the previous time window (Fig. 3c). However, the numbers of insect visits to west-facing, heated capitula during this time window are significantly lower than those observed for east-facing capitula. These data are consistent with our previous report that supplemental heating can partially but not fully rescue the number of insect visits to west-facing capitula (Atamian *et al.*, 2016). Together, these data indicate that temperature is an important, but not the only, determinant of pollinator floral preference in the morning.

We next examined the timing of pollen presentation on west-facing, heated capitula. Pollen is initially observed on these florets at ZT 1.50, approximately 30 minutes earlier than the time of first pollen release on unheated west-facing capitula (Fig. 4b). Differences in the fractions of florets releasing pollen on west-facing unheated and heated capitula are statistically significant between ZT 1.75 and ZT 2.75. In contrast, the fraction of florets presenting pollen on east-facing and west-facing, heated capitula is not significantly different at any time point (Fig. 4b). Thus, differences in capitulum temperature dynamics can fully account for the differential timing of pollen release in east- and west-facing capitula.

The kinetics of anthesis in sunflower is regulated by ambient temperature changes

To further understand the developmental basis for the effects of capitulum orientation and floral temperature on the timing of pollen emergence, we next investigated the effects of these factors on style and anther filament elongation, as development of these organs plays an integral role in the timing of pollen emergence (Lobello *et al.*, 2000; Sharma & Bhatla, 2013). Styles of east-facing capitula elongate rapidly between ZT 1.00 and ZT 2.00 (Fig. 5b), coinciding with the start of pollen emergence (ZT 1.25) on these capitula (Fig. 4b), while the rate of style elongation in west-facing capitula is slower (Fig. 5b). Since one conspicuous difference between east- and west-facing capitula is the elevated early morning temperatures observed on east-facing capitula (Fig. 5a, Atamian *et al.*, 2016), we tested whether temperature is responsible for the orientation-dependent differences in style elongation. We found that the kinetics of style elongation of east-facing capitula and those of artificially heated west-facing capitula are very similar (Fig. 5b).

To determine whether style growth parameters of east-facing, west-facing, and artificially heated west-facing capitula significantly differ, we applied Bayesian regression modeling to fit a Weibull growth model (Weibull, 1951; Yang *et al.*, 1978). The styles of untreated west-facing plants elongate more slowly than those of east-facing plants and artificially heated west-facing plants (posterior probability (pp) West < East growth rate = 0.9998; pp West < West heated = 0.9998; Supporting Information Fig. S6). Similarly, the inflection point of the style growth curve is later in west-facing plants as compared to east-facing plants and artificially heated west-facing plants (pp West > East inflection point = 0.9998; pp West > West heated = 0.9998; Supporting Information Fig. S6). The kinetics of style elongation are similar for east-facing and artificially heated west-facing capitula (Supporting Information Fig. S6). These data suggest that the easterly orientation of sunflower capitula at anthesis generates a temperature microclimate that promotes style elongation and pollen emergence so that they occur soon after dawn.

In contrast, the rates of anther tube emergence of florets on east-facing, west-facing, and west-facing heat-treated capitula appear very similar (Fig. 5c). Growth rates did not significantly differ between east- and west-facing plants (with or without heat) (pp West < East = 0.623; West heated < East = 0.432; Supporting Information Fig. S7). The inflection point of the anther growth curve is delayed both west-facing and artificially heated west-facing relative to east-facing capitula (pp West or West heated > E = 0.99); however there is no evidence that the heat treatment altered this parameter (pp West < West Heated = 0.492; Supporting Information Fig. S7). Thus, capitulum orientation and temperature have limited impact on the kinetics of filament elongation and anther emergence.

Because our field studies suggested small changes in ambient temperature regulate the rate of style elongation, we directly tested this possibility in an experiment conducted in an environmental chamber with all factors except temperature held constant. Just as in the field experiments, heated plants exhibit more rapid style elongation and earlier pollen presentation relative to unheated control plants (Fig. 5e and f). These results suggest that temperature regulates the timing of style elongation, which in turn determines the timing of pollen emergence from the anther tube. Overall, our results indicate that the elongation rates of styles and anther filaments are differentially sensitive to temperature and that temperature modulation of style elongation fine-tunes the timing of pollen presentation in natural conditions.

Discussion

As sunflowers approach anthesis and stem growth slows, daytime solar tracking movements from east to west slow until plants finally cease tracking altogether, resulting in east-facing capitula during the final stages of floret development. The circadian clock plays a critical role in regulating the sunflower tracking motion, and gating by the clock regulates the final easterly orientation of the capitulum (Atamian *et al.*, 2016). We previously found that east-facing plants receive more insect visits in the morning than west-facing plants, likely due to earlier warming of easterly oriented capitula (Atamian *et al.*, 2016). Many past studies have shown that plants have adapted different mechanisms to regulate flower microclimate including heliotropism, flower anatomy, and floral position (e.g., upward or downward orientation) (Corbet, 1990; van der Kooi *et al.*, 2019; Armbruster & Muchhala, 2020). Here we investigated whether capitulum orientation affects floret microclimate in sunflower and consequently influences anthesis, pollination, and seed development.

In many *Asteraceae* species including sunflower, style elongation drives pollen emergence by a plunger-type mechanism, with the stigma and style pushing pollen out the top of the anther cylinder (Putt, 1940; Lobello *et al.*, 2000; Sharma & Bhatla, 2013). An early report suggested that sunflower floret anthesis was slower under cooler conditions (Putt, 1940). Consistent with this, our experiments in controlled environments and the field show that the relatively small differences in temperature observed on east- and west-facing capitula are sufficient to increase the rate of style, but not anther filament, elongation to advance the phase of pollen presentation on east-facing capitula (Fig. 4 and 5). Although the molecular pathways by which warmer temperatures promote accelerated style growth are not yet known, sunflower homologs of *Arabidopsis* PIFs (PHYTOCHROME INTERACTING FACTOR) are attractive candidates. PIFs promote the growth of multiple *Arabidopsis* organs in a light-dependent manner; in addition, warm temperatures enhance the activity of some PIFs via multiple mechanisms (Paik *et al.*, 2017 Balcerowicz 2020). Intriguingly, some PIF proteins are both regulated by the circadian clock and help control clock entrainment, highlighting them as growth regulators that integrate external and internal cues (reviewed in Paik *et al.*, 2017; Creux and Harmer, 2019; Balcerowicz 2020). It is tempting to speculate that thermo-regulation of style elongation in sunflower may involve similar mechanisms; however, this remains to be determined. Intriguingly, sunflower anther filament elongation is strongly regulated by light and hormonal cues (Baroncelli *et al.*, 1990; Lobello *et al.*, 2000) but not by temperature in this study. Thus, late-stage development of anthers and styles are at least partially decoupled in sunflower via differential regulation in response to environmental cues.

We found that capitulum orientation influences fitness both through pollen- and seed-associated traits (Fig. 2 and 3b). East-facing capitula produce heavier, plumper seeds than west-facing capitula, but this effect is location-specific (Fig. 2 and S3). The location-specific nature of this finding is perhaps unsurprising as the climates of our two study sites (Davis, CA, and Charlottesville, VA) differ significantly, as did the total numbers of seeds produced by plants at the two sites (Supporting Information Fig. S1 and S2). Temperature differences between the sites may be responsible for the discrepancy in whether head orientation affected seed quality. It has been suggested that cloud cover and total amount of solar radiation received could impact sunflower seed development (Rawson *et al.*, 1984; Horváth *et al.*, 2020). We observed that Charlottesville had overall lower radiation than Davis and that the late summer planting in Davis had similar radiation exposure compared to peak season Charlottesville plantings (Supporting Information Fig. S1c). These differences may explain the locality specific differences we observed in seed traits and seed number (Fig. 2 and Supporting Information Fig. S2).

How might differences in capitulum temperature affect seed traits? In Davis, CA, we observed that the fronts of east-facing capitula reach a maximal temperature around noon and then cool down more rapidly than west-facing heads in the afternoon (Supporting Information Fig. S5a and S5b). In contrast, west-facing capitula reach maximal temperatures in the late afternoon, approximately five hours later than east-facing capitula (Supporting Information Fig. S5a and S5b). In Charlottesville, VA, differences in temperatures were still observed but were far less pronounced (Supporting Information Fig. S5c and d). Previous heat stress experiments in sunflower have shown that seed weight and filling are significantly negatively affected by extreme temperatures (Ploschuk & Hall, 1995; Rondanini *et al.*, 2006). It is possible that the higher afternoon temperatures observed on the fronts of west-facing capitula, particularly in Davis, CA (Supporting Information Fig. S5 and S8) could be detrimental to seed development. In addition, the circadian clock influences plant tolerance to heat shock and is closely associated with lipid metabolism pathways and ambient temperature response mechanisms (Hudson, 2010; Mizuno *et al.*, 2014; Kim *et al.*, 2019); the later phase of peak temperature on west-facing capitula compared to east-facing capitula may result in misalignment of this stress with clock-regulated heat response pathways, leading to reduced seed filling.

The greater number of insect visitations in the early morning to east-facing capitula likely explains their greater siring success compared to west-facing capitula (Fig. 3). This observation was made in both domesticated and wild sunflower populations (Supporting Information Fig. S4), suggesting that the easterly orientation of sunflower capitula at maturity predates the domestication of sunflower. Previous work has shown that the position of bilaterally symmetrical flowers such as honeysuckles and snowdrops can affect interactions with pollinators (Giurfa *et al.*, 1999; Fenster *et al.*, 2009; Xiang *et al.*, 2020). In *Nicotiana attenuata*, flower angle changes throughout the day and is influenced by circadian clock genes. Mutations in clock genes led to changes in floral angles, causing possible pollinator shifts (Yon *et al.*, 2016; Yon *et al.*, 2017a; Yon *et al.*, 2017b). Although it has been suggested that individual flower orientation is not an adaptive trait for radially symmetrical flowers like sunflower (Armbruster & Muchhala, 2020), our findings suggest that this is not always the case. Furthermore, our observation that east-facing flowers have more visitors than west-facing flowers for only a relatively short period of time (Fig. 3c) suggests that even a small shift in the daily timing of pollinator visitation can enhance relative siring success.

What might account for this time-of-day specific difference in insect visits to east- and west-facing capitula? Our data support roles for both temperature-dependent and temperature-independent factors, as proposed by van der Kooi (2016). Although artificially heated west-facing capitula receive more pollinator visits than unheated west-facing capitula, they are not visited as often as east-facing capitula during the period after dawn (Fig. 3c). Illumination by incident light is one obvious non-thermal and time-of-day-specific difference between east-facing and west-facing capitula: easterly oriented capitula are in full sun at first light while west-facing plants remain shaded. Sunflower petal adaptations, such UV markings have recently been shown to facilitate pollinator visits (Todesco *et al.*, 2021), and it might be expected that these would be more visible on capitula directly facing the sun compared to those facing away. Indeed, we found that in the morning east-facing capitula are more visible than west-facing capitula, appearing brighter yellow and having more clearly visible UV markings (Supporting Information Fig. S9). In some species, bright light can also trigger release of floral volatiles (Hu *et al.*, 2013); if also true in sunflower, a difference in timing of this response might also help explain the greater early morning insect visits to east-facing capitula.

There are also several possible explanations for our finding that artificial warming promotes insect visits to west-facing capitula (Fig. 3c). The approximately 30-minute phase advance in both the timing of insect visits and pollen release on heated compared to unheated capitula (Fig. 3c and 4b) suggests that temperature-dependent changes in the timing of floral development may play an important role in promotion of insect visits. One possibility is that earlier release of pollen rewards on east-facing capitula directly promotes increased insect visits, as suggested in other studies (Engel & Irwin, 2003; Muth *et al.*, 2016; Nicholls *et al.*, 2016). This hypothesis is supported by the general correlation between the time pollen presentation is first observed and the time frames when insect visits to capitula are most frequent (Fig. 3c and 4b).

A precise correlation between pollen emergence and insect visits is not expected, since bee behaviour is determined not only by floral rewards but also by the insect circadian clock (Bloch *et al.*, 2017). Even after only one day of training with exposure to food rewards at a specific time of day, foraging bees often arrive at the feeding station well before the expected feeding time (Moore *et al.*, 1989; Moore, 2001). Thus, pollinators may have begun visiting the east- and west-facing capitula in our study in anticipation of the release of floral rewards such as pollen. It is also possible that other temperature-dependent floral developmental traits that we did not measure, such as release of volatiles (Hu *et al.*, 2013; Sagae *et al.*, 2014), may contribute to the advanced phase of insect visits to warmer capitula. Finally, pollinator behaviour can also be directly affected by floral temperature (Sapir *et al.*, 2005; Rands & Whitney, 2008; Norgate *et al.*, 2010). Exothermic pollinating insects may simply prefer the heat reward that warmer surfaces offer in the morning, independently of the timing of floral developmental traits. Overall, we consider it likely that multiple factors, including temperature-dependent differences in the timing of floret development, promote insect preference for east-facing versus west-facing capitula in the morning.

While we have demonstrated a correlation between the timing of anthesis and reproductive success in an outcrossing species, small differences in the timing of anthesis have been shown to affect reproductive success is self-pollinating plants as well. In rice, advancing the time of flower opening in the morning by as little as 90 minutes has been shown to improve yield by allowing plants to complete fertilization before the onset of heat stress in the middle of the day (Hirabayashi *et al.*, 2015). Thus, further investigation into the pathways regulating the daily timing of anthesis in crops is likely to be of general agronomic importance.

In conclusion, we show that the typical easterly orientation of the sunflower capitulum produces a specific daily temperature dynamic that influences the microclimate of the florets and the precise timing of pollen emergence. We demonstrate that the phase of pollen release is controlled by temperature-dependent changes in the rate of style elongation (Fig. 5g) and that modulation of this phase affects the timing of pollinator visits to flowers. Finally, we show that the natural eastward orientation of capitula, imposed by complex interactions between the plant circadian clock and environmental cues (Atamian *et al.*, 2016), has important implications for female and male fitness-related traits. Thus, our results demonstrate that the easterly orientation of mature sunflower capitula plays an important role in managing the floret microclimate and ensuring the correct conditions for anthesis, pollination, and seed development.

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Author Contribution

NMC, EAB, BKB, and SLH planned and designed the research. NMC, EAB, AGG, SS, CLS, SVH, DY, and BKB conducted fieldwork and performed experiments. JNM performed Bayesian analysis; all authors contributed to other data analyses. NMC, BKB, and SLH wrote the manuscript, with contributions from all authors.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Fig. 1. Sunflower capitulum morphology and floret development. Sunflower capitulum showing the outer ray florets (RF) and the centripetal development of the inner disk florets (DF), which mature on a daily basis in a stepwise fashion from the outside to the inside in concentric pseudowhorls (left panel). Enlarged images of florets (top right) show the three or four rings of florets in each pseudowhorl at each distinct developmental stage: immature florets, staminate florets with elongating styles pushing pollen out of the anther tubes (A), and pistillate florets with receptive stigmas (S) emerging from the fused anther (A) tubes (bottom right).

Fig. 2 Sunflower capitulum orientation affects multiple seed traits. Capitulum diameter (a), dehulled seed width (b), and median seed weight (c) (a proxy for filling) from east-facing flowers (red) and west-facing flowers (blue) collected in field trials over three consecutive years. Linear models with trial and year as random effects and direction as a fixed effect show that orientation significantly affects all three traits. Box edges represent the 75th and 25th percentile, box midline represents the median, and whiskers represent the largest or smallest value within 1.5 times the interquartile range. ** $P < 0.01$, *** $P < 0.001$.

Fig. 3 East-facing capitula have a siring advantage and attract more insects early in the morning. (a) Schematic of experimental design, with cytoplasmic male sterile (CMS) female sunflowers in the center and two male fertile genotypes surrounding them with sire genotype A (SA) facing east and sire genotype B (SB) facing west. (b) Number of offspring sired by east-facing (red) and west-facing (blue) plants. Three different sire genotypes (S1 = RHA279, S2 = R-188, and S3 = RHA397) were used and paired in different SA-SB combinations in seven trials. Reciprocal tests with the orientations of the sire genotypes switched were performed with the same sires for two of the three possible sire genotype combinations over these trials. For each trial, offspring sired by each paternal genotype were counted, and * = $P < 0.05$ or ** = $P < 0.01$ for chi-square test with 1 df. (c) Average number of insect visits over a 20-minute period to east-facing (red bar), west-facing (blue bar), and west-facing and heated (orange bar) sunflower capitula at several time points in the morning. Letters represent treatments with significantly different means (P <0.05; one-way ANOVA with multiple pairwise comparisons carried out using Tukey's HSD). (d) Temperature of east-facing (red line), west-facing (blue line) and west-facing and heated (orange line) capitula. ZT, Zeitgeber time, with ZT0 representing sunrise. Error bars represent SEM; shaded regions indicate 95% confidence intervals.

Fig. 4: Sunflower capitulum orientation and temperature affect timing of morning pollen release. (a) Representative images of sunflower florets on east- and west-facing flower heads with arrowheads indicating specific florets at different time points (b) Percent of florets releasing pollen on field-grown east-facing (red), west-facing (blue), and west-facing and heated (orange) flower heads. “a” and “b” indicates time points with significant differences between east-facing, west-facing and west-facing heated plants within each time point using a two-way ANOVA with multiple comparisons for factors time and capitulum orientation ($P < 0.05$). Error bars represent the SEM (n=6). ZT, Zeitgeber time, with ZT0 representing sunrise.

Fig. 5. Sunflower capitulum temperature affects the rate of style elongation and the timing of pollen emergence in the field and in controlled conditions. (a - c) Field-grown plants. (a) Temperatures of east-facing (red), west-facing (blue) and west-facing plus heated (orange) sunflower capitula were monitored (note that the temperatures of the east-facing and west-facing plus heat capitula are indistinguishable). (b) Style length dynamics of east-facing, west-facing and west-facing and heated florets. (c) Combined length of floret and emerging anther tube was measured as a proxy for anther filament length over time. (d – f) Plants grown in controlled environment chambers. (d) Temperatures of sunflower heads with (red) and without (blue) supplemental heating, n = 2. (e) Style length dynamics in the florets of sunflower heads treated with (red) and without (blue) supplemental heating. (f) The percent of anthers releasing pollen on heat-supplemented (red) or control (blue) flowers. Two-way ANOVA for time and temperature factors. * indicates $P < 0.05$. ZT 0 indicates dawn/lights on. For panels a – e, Loess functions were fit to data collected at 15 min intervals; grey areas represent 95% confidence intervals. (g) A schematic representation of the effect of temperature on the kinetics of style elongation and pollen emergence, where higher temperatures shorten the time taken for full pollen release. Double arrowheads indicate the time points where full elongation of the style is reached, with red indicating heated styles reaching full elongation sooner than unheated styles (blue).

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Comparison of average temperatures and solar radiation in Davis, CA and Charlottesville, VA.

Fig. S2 No difference in the average seed number between east- and west-facing capitula in Davis, CA and Charlottesville, VA.

Fig. S3 No difference in seed weight or width between east- and west-facing capitula in Charlottesville, VA.

Fig. S4 Capitulum orientation affects the timing of insect visitation and pollen presentation by wild sunflower plants.

Fig. S5 Average temperature changes on the east- and west-facing sunflower capitula over a 24 hr period in Davis, CA and Charlottesville, VA.

Fig. S6 Bayesian modeling of style growth.

Fig. S7 Bayesian modeling of anther growth.

Fig. S8 Capitulum orientation has larger effects on floral temperature in Davis, CA than in Charlottesville, VA.

Fig. S9 Capitulum orientation in the morning changes sunflower visual aspects in both the visible and UV ranges of the spectrum.

Table S1 Summary table of experiments and measurements.

Table S2 Detailed statistical analysis of siring success of differentially oriented *Helianthus annuus* cultivars.

Methods S1 Detailed description of plant growth conditions in field and in controlled environment chambers.

Methods S2 Experimental methods for siring experiments.

Methods S3 Bayesian modeling of anther and style growth

References References for Supporting Information

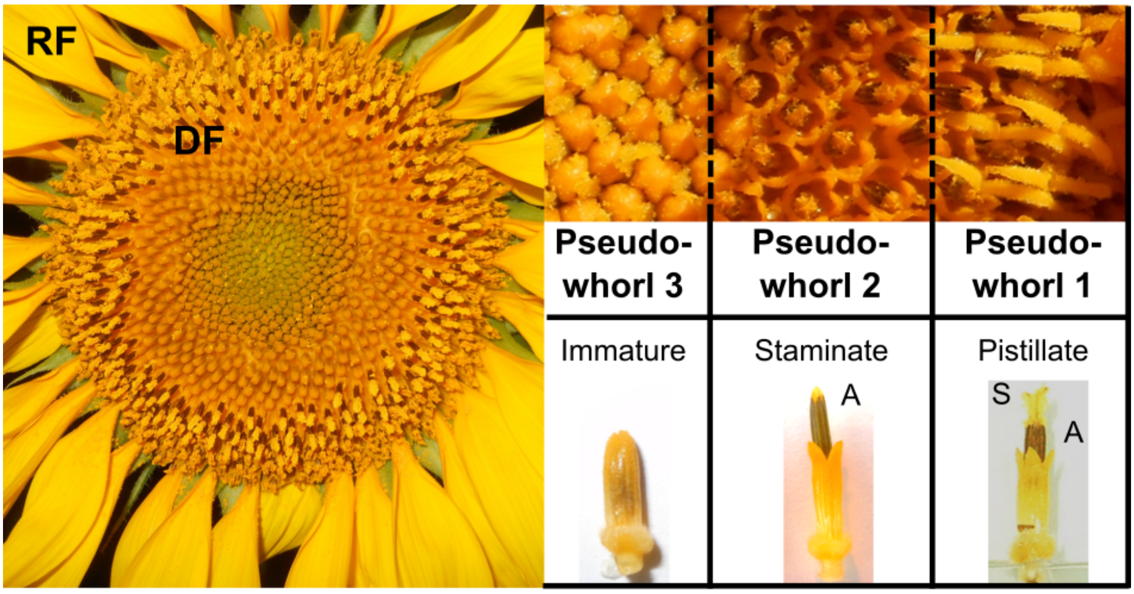


Fig. 1. Sunflower capitulum morphology and floret development. Sunflower capitulum showing the outer ray florets (RF) and the centripetal development of the inner disk florets (DF), which mature on a daily basis in a stepwise fashion from the outside to the inside in concentric pseudowhorls (left panel). Enlarged images of florets (top right) show the three or four rings of florets in each pseudowhorl at each distinct developmental stage: immature florets, staminate florets with elongating styles pushing pollen out of the anther tubes (A), and pistillate florets with receptive stigmas (S) emerging from the fused anther (A) tubes (bottom right).

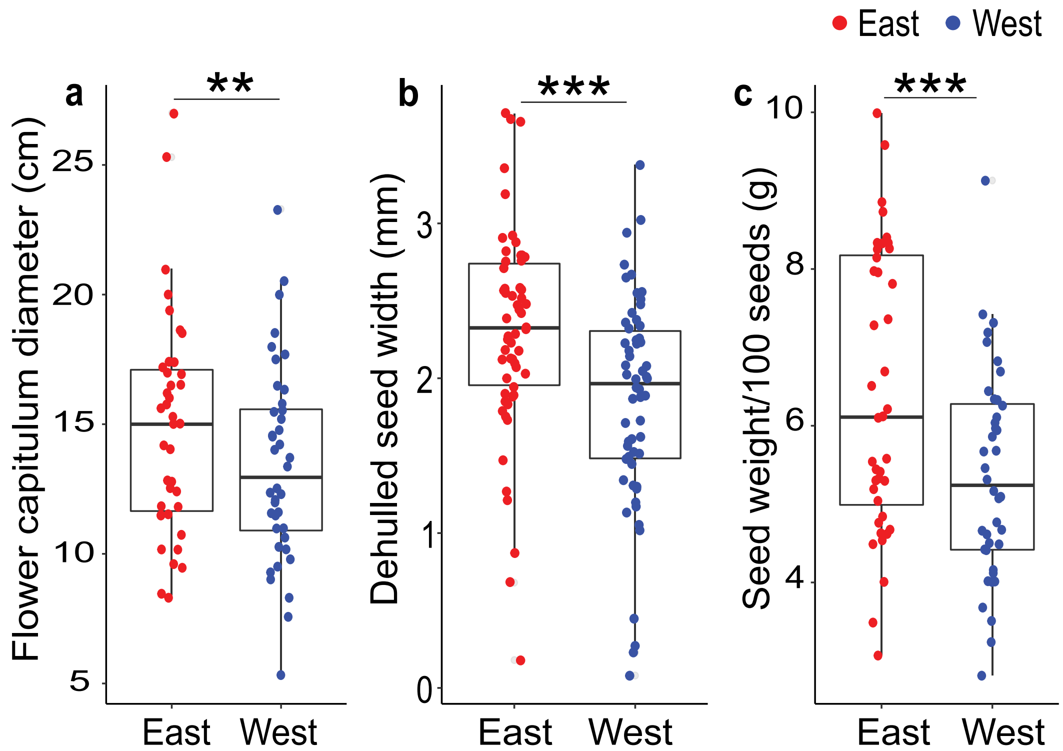
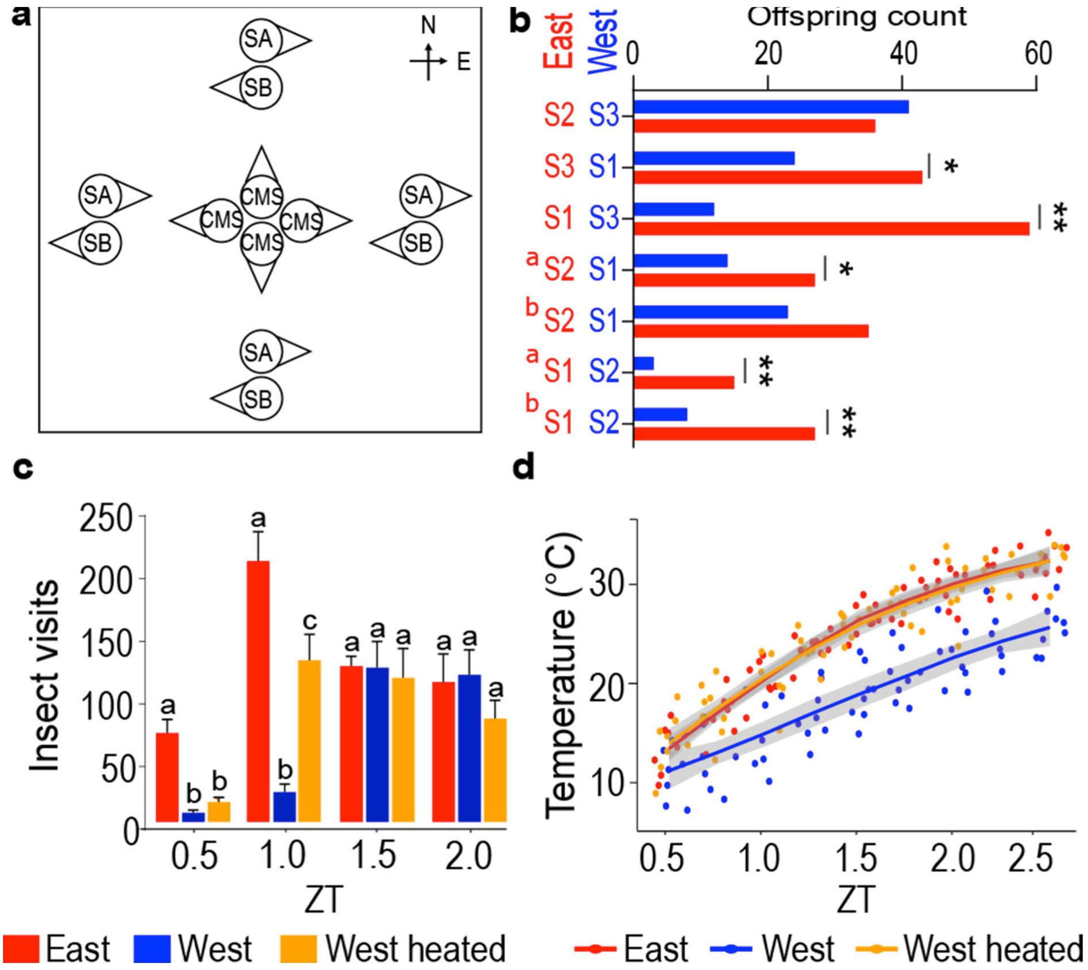


Fig. 2 Sunflower capitulum orientation affects multiple seed traits. Capitulum diameter (a), dehulled seed width (b), and median seed weight (c) (a proxy for filling) from east-facing flowers (red) and west-facing flowers (blue) collected in field trials over three consecutive years. Linear models with trial and year as random effects and direction as a fixed effect show that orientation significantly affects all three traits. Box edges represent the 75th and 25th percentile, box midline represents the median, and whiskers represent the largest or smallest value within 1.5 times the interquartile range. ** $P < 0.01$, *** $P < 0.001$.



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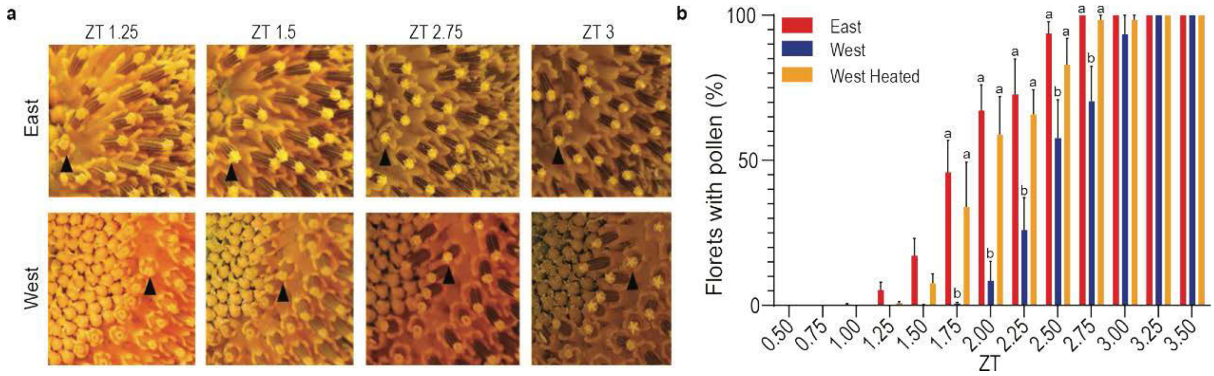

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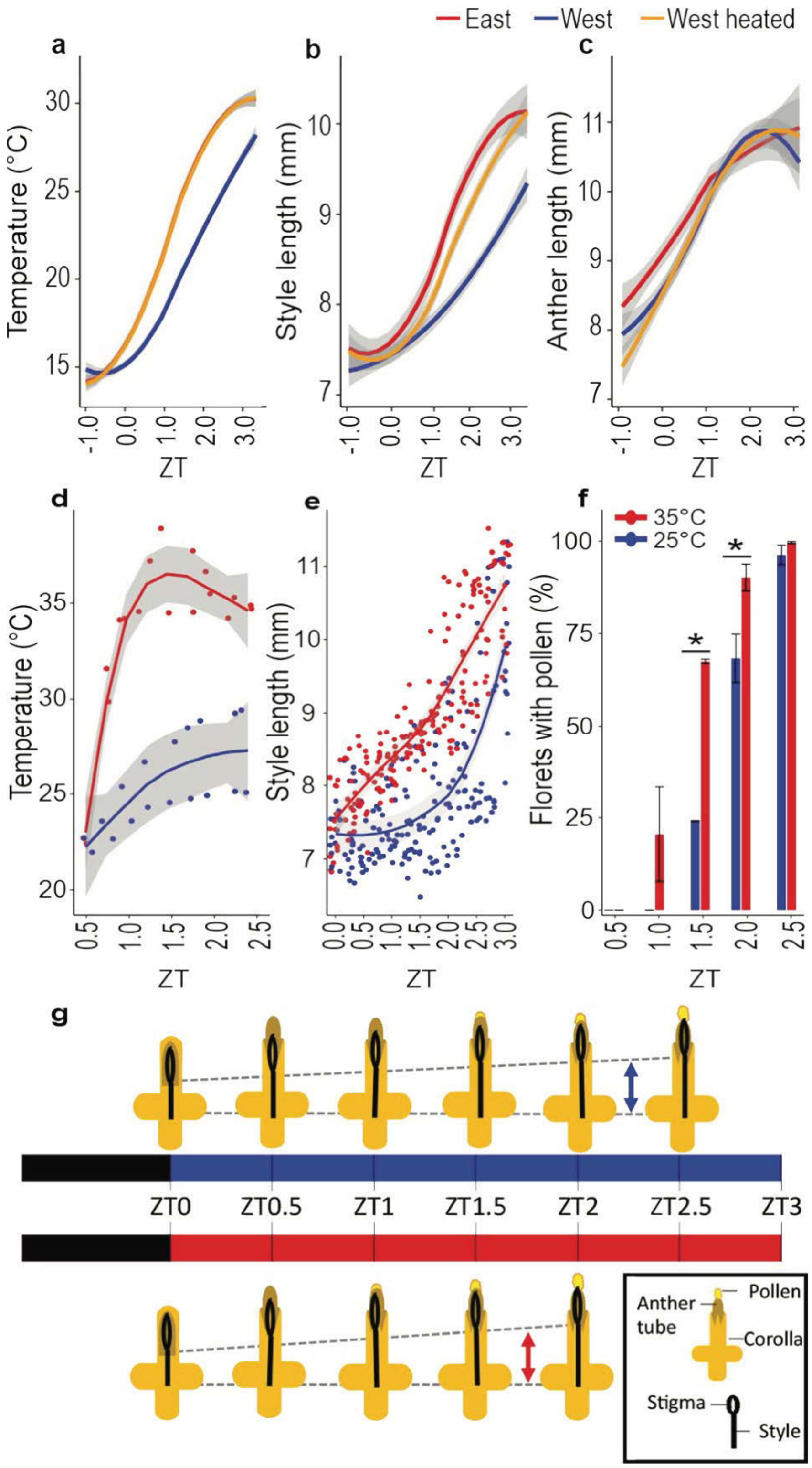

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