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The Effects of Extreme Heat and Heat Waves on the Ecology of Pollination and Floral Reproduction

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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DAVIS

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Abstract

Climate change and climate instability driven by anthropogenic fossil fuel emissions are increasing the frequency and severity of extreme weather events. One such event, heat waves, are becoming more frequent, intense and prolonged (Meehl and Tebaldi 2004; Stillman 2019; Thiery *et al.* 2021). Extreme heat associated with these heat waves may be particularly damaging to critical species life-history events such as reproduction and mating, and also interactions between organisms (Vasseur *et al.* 2014; Harvey *et al.* 2023). Heat may especially impact plant-pollinator interactions and the mating of flowering plants, as heat may disrupt the process of pollination and alter the stable dynamics of mating (Hedhly *et al.* 2009; Walters *et al.* 2022; Hemberger *et al.* 2023). Importantly, heat may disrupt plant-pollinator interactions and successful mating directly through heat's effects on flower gamete production and performance (Hedhly *et al.* 2009), but also indirectly through heat's effects on foraging pollinators (Walters *et al.* 2022; Hemberger *et al.* 2023). My dissertation is divided into three chapters with the aim of understanding methodically how heat impacts the ecology of pollination and floral reproduction. Chapter 1 used experimental pollination treatments explicitly focusing on the perspective of the plant to understand how heat affects the dynamics of pollen limitation-the reduction in potential seed production through insufficient receipt of or pollen that is incapable of fertilizing ovules. It evaluates how heat may simultaneously influence the quantity of pollen grains produced and the quality of those pollen grains in addition to pollen quality effects inherent to pollen origin (self versus outcross). I found that heat dramatically limited pollen production, and the quality of pollen grains measured as its viability and performance to grow pollen tubes and fertilize ovules. Although outcross pollen was of higher quality than self pollen under control conditions, heat diminished these differences. These results suggest that heat primes plants to have a high risk of quantitative and qualitative pollen limitation and can disrupt plant mating strategies. Chapter 2 used experiments to quantify the relative contributions of direct (through flowers) and indirect (through pollinator foraging and flower visitation) effects of extreme heat on pollination, post-pollination, and reproduction. The experiments involved bumble bee foraging in heated chambers to allow bumble bees to disperse pollen between flowers. I found that heat strongly limited pollination, the success of those dispersed pollen grains (post-pollination) and reproduction through direct and indirect effects, and that direct effects of heat in post-pollination caused outright reproductive failure. Importantly, these effects were additive. Although both direct and indirect effects are highly limiting, the presence of direct effects dominated heat's indirect effects on pollination, post-pollination, and reproduction. Chapter 3 used mathematical modelling to explore the sensitivity of pollination and plant reproduction to heat based on models of pollination dynamics and thermal performance curves. Due to logistical challenges that emerge in experimentation, mathematical models allowed me to overcome them and fully explore the parameter space to understand which temperatures specifically would be deleterious, and whether this depended on direct and indirect effects. Furthermore, this chapter also explored whether altering the density of pollinators could offset the negative effects of heat on flowers for pollination and post-pollination processes. My results demonstrated that pollination and reproduction respond non-linearly at high temperatures as pollination and reproduction abruptly collapse. This collapse was largely driven by decreasing pollen production, and therefore increasing pollinator abundance did not compensate for how flowers respond to heat. The collection of these chapters builds upon previous work that suggests that pollination should be highly vulnerable to heat. My results underscore the broad detrimental effect that heat has at each stage of the reproductive process (pollination and post-pollination), and that there is little to no

resilience in pollination systems to the effects of heat. When plants mate during periods of extreme heat, it will likely severely limit the reproduction of plants through direct and indirect pathways (Walters *et al.* 2022; Hemberger *et al.* 2023), which threatens the persistence of plant populations and the security of food production (Battisti and Naylor 2009; Lesk *et al.* 2016).

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Chapter 1: Extreme heat increases risk of pollen limitation through reductions in pollen production and pollen quality

Introduction

Heat waves are becoming more frequent, intense, and prolonged as climate warming increases global average temperatures and drives climate instability (Meehl and Talbaldi 2004; Stillman 2019; Thiery *et al.* 2021). These amplified heat waves expose organisms to high temperatures that may disrupt sensitive life history events as well as alter their interactions with other organisms (Jamieson *et al.* 2012; Pincebourde and Casas 2019) by pushing them beyond their thermal optima (Harvey *et al.* 2023). One particular life history event that is highly sensitive to extreme heat is the floral reproduction of angiosperms (Hedhly *et al*. 2009). When heat waves coincide with flowering, high temperatures may disrupt floral development and alter the dynamics of pollination (Nicholson and Egan 2020), successful plant reproduction (Hedhly *et al.* 2009; Hedhly 2011), plant demographics (Jiménez *et al.* 2011), and global food production (Battisti and Naylor 2009).

Extreme heat during floral development can impact gametogenesis, reducing the quantity of pollen produced and its quality (Hedhly *et al.* 2009; Zinn *et al.* 2010). These effects combine to reduce the availability of pollen that can be dispersed to receptive stigmas and the potential for those successfully-dispersed pollen grains to fertilize ovules that will become seeds. Extreme heat can also impact pollen receipt by compromising stigma receptivity where pollen is deposited, and reducing fertilization by impeding pollen tube growth within the style (Prasad *et al.* 2002; Hedhly *et al.* 2005, 2009; Hedhly 2011; Distefano *et al.* 2018). Although previous studies (predominantly using crop plants) have demonstrated that extreme heat causes deleterious responses in flowers, such responses have not been integrated into the framework of floral reproductive ecology and how relative effects of heat on pollen availability and pollen vigor lead to pollen limitation.

Pollen limitation occurs when seed production is limited by pollen receipt instead of resource availability (Ashman *et al.* 2004; Knight *et al.* 2005). This limitation develops via two general pathways, referred to as quantitative pollen limitation and qualitative pollen limitation (Aizen and Harder 2007; Harder and Aizen 2010). Quantitative pollen limitation occurs when an insufficient number of pollen grains are received on stigmas to fertilize the available ovules that a plant has and is able to otherwise mature into seeds. Under normal environmental conditions, qualitative pollen limitation arises from reduction in pollen vigor due to pollen origin and genetic relatedness to the maternal plant (Kao and McCubbin 1996; Jóhannsson *et al.* 1998; Souto *et al.* 2002). Thus, pollen from different donor plants varies in quality both absolutely and as it relates to the recipient plant. Self-pollination represents the extreme of pollen donor and recipient relatedness and often the lowest vigor. Because of differences in vigor (hereafter quality), outcross pollen is more likely than self pollen to germinate pollen tubes into stylar tissues, and those pollen tubes are more likely to successfully grow the length of the style to allow for fertilization (although exceptions exist in highly inbred populations: Jóhannsson *et al.* 1998; Aizen and Harder 2007; Harder and Aizen 2010). Such qualitative differences in fertilization success between outcross and self pollen mean that individual flowers need to receive more self pollen grains relative to outcross pollen grains for complete ovule fertilization.

Regardless of pollen origin, pollen tubes compete for limited space available within styles and this competition intensifies at higher levels of pollen deposition (Cruzan 1986, 1989; Harder, Aizen, Richards, *et al.* 2016; Harder, Aizen, and Richards 2016), creating a non-linear relationship between pollen deposition and pollen tube survival (Aizen and Harder 2007). This non-linear relationship is an essential feature of the post-pollination process, and the link between pollen deposition and seed set. Because of this non-linear relationship, differences in pollen tube survival between cross and self-pollination are most evident from low to intermediate levels of pollen deposition, but their relative effects diminish when pollen deposition is high (Figure 1). Thus, quantitative pollen limitation tends to occur at lower levels of pollen receipt (insufficient pollen receipt) and qualitative pollen limitation tends to occur at intermediate pollen receipt (pollen origin affects quality).

Although both quality and quantity limitation exist under normal environmental conditions, pollen production and pollen quality can additionally be impacted by extreme heat. Heat-induced decreases in pollen quality should further limit pollen grain success and exacerbate pollen limitation arising from pollen origin (self vs. outcross). Under normal conditions, origin-based differences in pollen quality govern the amount of pollen required to overcome pollen limitation, and this requirement should be higher from self pollen (Figure 1). However, when flowers develop under heat stress the negative effect on pollen quality may add to differences based on pollen origin (Figure 1) and increase pollen limitation. These potential pollen quality impacts inform our expectations of how flowers respond post-pollination, and our expectations of overall pollen limitation risk from heat and pollen origin. However, extreme heat may also exacerbate quantitative pollen limitation by reducing how much pollen each flower produces.

Reduced pollen production by flowers exposed to heat during development should diminish the pool of pollen that is available to be dispersed among receptive flowers and thus the amount of pollen deposited on receptive stigmas on average (Cresswell 1999). For some species, quantitative pollen limitation can be overcome through self-compatible mating systems that supply additional viable pollen grains originating within each plant, albeit with some risk of qualitative pollen limitation (Harder *et al.* 2007; Barrett and Harder 2017). However, under periods of heat stress, this reproductive assurance afforded by abundant self-compatible pollen may be lost because of reduced pollen availability.

Considered together, the effects of extreme heat on pollen quality and pollen availability likely compound the risk of pollen limitation during heat waves and impose both quantitative and qualitative pollen limitations. Identifying these implications is an essential piece to understanding how both wild plant populations and fruit-bearing crops will be affected by ongoing climate change. We tested the effects of simulated heat waves on *Brassica napus* (a self-compatible annual plant) to ascertain how extreme heat affects pollen quality and quantity limitation and reproductive success. From our framework, we predict that:

1. Extreme heat imposes qualitative pollen limitation by reducing the quality of pollen grains and increasing the number of pollen grains needed to overcome pollen limitation.
2. This effect of extreme heat on pollen quality and pollen limitation should be additive to differences in pollen quality that already exist between outcross and self-pollination.
3. Extreme heat should decrease the number of pollen grains produced per flower, and thus increase pollen limitation through reduction in total pollen available for dispersal and deposition onto stigmas.

Methods

Experimental heat treatments

We tested our predictions experimentally by exposing *Brassica napus* plants to experimental heat waves in growth chambers. We collected anthers to measure pollen production and performed hand pollination using outcross and self pollen on plants from the same experimental temperature treatments to assess extreme heat effects on pollen quality and pollen limitation. We chose *B. napus* as a focal test species due to its self-compatible mating system, known temperature sensitivity (Young *et al.* 2004), and importance as a widely grown crop plant. We used seeds of rapid cycling *B. napus* sourced from the Rapid Cycling Brassica Collection University of Wisconsin (Stock #W5-1), which are not inbred lines and retain considerable genetic diversity (P. H. Williams pers comm.).

We seeded cohorts of plants in Vigoro All-Purpose Potting Mix (Vigoro, Lake Forest, Illinois, USA) in 4-inch pots and initially grew them inside under full spectrum lights fluorescent lights between February and March 2021. Once their first true leaves had developed we moved plants to a greenhouse where they were kept at 25C on a 16/8 day/night cycle, watered daily, and fertilized every 3 days with Peters Professional Fertilizer with micronutrients at 1:1:1 - N:P:K at 100 ppm volume. When plants began to develop branching racemes, they were moved into treatment chambers for experimental heating during April and May 2021.

From each cohort (n=20) we placed 9 plants in an experimental heating chamber and 9 plants into a replica control chamber for the temperature treatment trial (Figure S1). Plants were kept in their respective treatment chambers for 72 hours and under the same day night cycle with full spectrum fluorescent lights. In the heating chamber, ceramic heat lamps were used to elevate daytime temperatures to ~35C to simulate heat wave conditions. During the night cycle, the heating elements were turned off. We chose this temperature because it is at the edge of thermal tolerance for *B. napus* (Young *et al.* 2004), and represents a moderate heat wave in its growing regions. After the treatment period, we collected the anthers of one flower from each plant to measure pollen production (see below).

Hand pollination experiments

We conducted hand pollination experiments after temperature treatments on emasculated virgin flowers to measure pollen limitation and effects of heat on outcross and self pollen success. We purposefully varied the amount of pollen transferred across replicates in order to capture the asymptotic nature of deposition-pollen tube growth relation (Figure 1). Emasculation precluded the possibility of self pollen grains being deposited on the stigmas of cross-pollinated flowers. In order to quantify the impacts of pollen origin on pollen quality within the context of heat waves, plants that had either been exposed to heat or normal temperatures were hand pollinated using either outcross pollen or self pollen. Outcross pollen was always from donors in the same trial so that heat-stressed recipient plants were pollinated by heat-stressed donor plants and control plant recipients were pollinated by control plant donors. When possible, we pollinated two virgin flowers on each plant, one was given cross-pollination and another to be self-pollinated so that differences among plants could be accounted for. To conduct cross-pollination, we put anthers from 3 plants (one flower per plant) into an Eppendorf tube and shook it to mix the pollen. We then used a cotton swab to pick up pollen and deposit it on the stigma of the virgin flower. We repeated this with a fresh Eppendorf tube for self-pollination but used anthers from 3 self-originating flowers. We visually inspected stigmas during hand pollination to categorize the level of pollen deposition. After hand pollination was complete, the flowers were left for 24 hours to ensure pollen tube growth was complete (Young *et al*. 2004). We then collected styles and stored them in 1.5ml Eppendorf tubes filled with 70% Ethanol.

Pollen deposition and pollen tubes

To prepare style samples for staining, we rinsed them with DI water and then left them to soak for 1 hour before being transferred to 8M NaOH solution. Once into the NaOH solution we heated them on a hot plate for 1 hour at 55C to soften style tissues. We then rinsed styles again with DI water and soaked them for 1 more hour before moving them to a buffer of K_3PO_4 0.1M with 1% aniline blue dye for staining (mixed at 90% K_3PO_4 and 10%, 1% aniline blue). We then kept styles in the refrigerator for 24 hours to stain thoroughly before squashing them on microscope slides for observation under a fluorescent microscope (Martin 1959). Under fluorescent light we counted the number of pollen tubes at the base of the style, and the number of pollen grains deposited on the stigma. Although some pollen tubes may not have fluoresced, this should not be biased between treatments. These measurements together inform at the individual flower level how much pollen is required to overcome pollen limitation, and the effects of heat and pollen origin on pollen quality.

Pollen production

After temperature treatments, anthers from a single flower on each plant were taken to assess the impacts of heat stress on pollen production. We placed anthers from flowers in 70% ethanol inside 1.5 mL Eppendorf tubes for later processing and counting. Flowers were selected haphazardly at different ages of open flowers to represent plant level pollen availability after 72 hours of heat exposure. Importantly, we acknowledge that flowers that opened at the onset of the simulated heat wave would have already developed pollen grains, and so we may underestimate the full effect of heat on pollen production. Our estimate reflects availability of pollen per flower during a modest duration heat wave.

We used a Coulter Multisizer 3 Particle Counter (Beckman Coulter, Brea, California, USA) to count pollen grains from anthers that had been stored in 70% ethanol. First, we vortexed Eppendorf tubes containing anthers, transferred their contents into a coulter cuvette and sonicated them using a Microson^{TM} Ultrasonic Cell Disruptor (Misonix, Farmingdale, New York, USA) to release any residual pollen grains within anthers. We then rinsed individual anthers into the cuvette with 0.9% saline solution. For each cuvette, we weighed the empty weight of the cuvette and the final weight of the cuvette to calculate sample volume. We counted the number of particles between 20-30 um within three 1 mL of solution. We averaged the number of pollen grains from the three samples and multiplied it by the weight of the sample to calculate total pollen grains per flower.

Seed collection

We collected fruits from hand pollination experiments at fruit maturity and when siliques were beginning to yellow and placed them in small coin envelopes. After the fruits had completely dried, we counted the seeds from each fruit. We collected all fruits that had been hand pollinated in the experiment and counted the number of developed seeds in each fruit to measure the seed set.

Data analysis

We conducted all analyses in R (v4.3.1). We used the package nlme (Pinhiero *et al.* 2023), and developed nonlinear mixed models to analyze the relationship between pollen deposition and pollen tubes reaching the base of the style, building on a previously documented functional form (Aizen and Harder 2007). We used the package glmmTMB (Brooks *et al.* 2023) to analyze pollen production and seed set as general linear models, and generalized linear mixed models, respectively.

Pollen quality- Previous work using other species from different angiosperm families documented a consistent asymptotic relationship between pollen deposition on stigmas and pollen tubes reaching the base of styles (Aizen and Harder 2007; Harder, Aizen, and Richards 2016). Like in those previous explorations, we modelled pollen tube survival as a decelerating asymptotic function of pollen deposition (Equation 1) where T is the number of pollen tubes surviving to the base of the style, α is the asymptote, r is the rise of the function, and P is the number of pollen grains on the stigma.

$$T = \alpha\,(1 - e^{-r * P}) \qquad \text{eq 1.}$$

We fit this functional form to our data and compared the various parameters among our temperature and pollen origin treatments. We used likelihood ratio tests between three nested models to test our hypotheses about the impact of heat and pollen origin on pollen quality. The null model considered pollen tubes reaching the base of the style only as a function of pollen deposited on stigmas, the second a single factor temperature treatment model (control and heat), and the third a two-factor temperature*pollen origin model. All three models included temperature treatment trial as a random effect rather than plant because per plant observations consisted of only 1-2 flowers. This temperature*pollen origin model estimates an individual rise (r) parameter for each level of the temperature and pollen origin interaction. In our models we chose to fix the asymptote (α) to a value estimated solely from outcross control plants and then set this limit ($\alpha = 25.8$). This choice reflects a conceptual understanding of pollen deposition on stigmas and also analytically allows for a clearer interpretation of heat and origin impacts (in framework of Figure 1). Conceptually, the choice suggests that, with sufficient deposition, the number of pollen tubes from lower quality sources reaching the base of the style will eventually converge on that from high quality sources if sufficient pollen is available. We recognize that stigma clogging from low quality pollen could disrupt this accumulation (Snow *et al.* 2000; Barrett 2002). Using a fixed asymptote assumes that the effect of extreme heat is only influencing pollen quality and that pollen tube survival is not being affected by heat at the stylar level, which is in line with our experimental design (pollination after temperature treatment). Analytically, having an independent rise and asymptote in the model also would have required the maximum likelihood estimation to solve for both simultaneously. Because of the nature of this estimation, solving simultaneously may cause one parameter to overpower the other parameter when looking for the best fit and mask its effect. Therefore, we used a fixed asymptote so that we could clearly identify the effects of our treatments on pollen quality via the rise (r) parameter. For model fitting we also constrained observations to those where there were 400 or less pollen grains deposited on the stigma. We constrained pollen depositions values to < 400 because it encompassed the range where qualitative effects emerge and represented 75% of all observations. In the control treatments pollen deposition ranged from 2-1225 grains and in the control treatments 0-561 grains. Constraining the model also aided model convergence. We used pairwise comparisons based on the temperature*pollen origin model between *r* parameter estimates to assess statistical effects between temperature and pollen origin effects. Comparisons between the *r* estimates allowed us to address pollen quality explicitly under the assumption that the treatments shared similar limits to pollen tube survival.

Pollen quantity- To test for differences in pollen production between heat and control plants, we used a general linear model assuming a normal distribution for the number of pollen grains per flower. We included temperature treatment as a fixed effect such that differences in pollen grains per flower indicates the effect of the temperature treatment (control vs. heat).

$$pollen\ grains\ per\ flower \sim temperature$$

Because each observation for this analysis came from an individual plant, we do not include a random effect as observations are assumedly independent.

Seed set- To test differences in seed set from heat and control plants, and cross and self-pollination, we fit a generalized linear model assuming a negative binomial distribution, with an interaction between temperature treatment and pollen origin. We included temperature treatment trial as a random effect for the same reason as stated in the pollen quality section.

$$seed\ set \sim temperature\ *\ pollen\ origin\ +\ (1|\ trial)$$

From this model we estimated marginal means using the emmeans package (Lenth *et al.* 2023), and used pairwise comparisons to compare differences in means between treatments. Differences between means in the pairwise comparisons indicate the relative effects of pollen origin (outcross vs. self), and temperature treatment (heat vs. control) on seed production. We also examined model residuals and used dispersion tests using the DHARMa package (Hartig and Lohse 2022) to assess model fit.

Results

Pollen origin and temperature significantly affected pollen quality. Comparing among models of the relation between pollen deposition and pollen tubes, the model that included temperature treatment better explained variation than the null model (Table 1a, 1b $P<0.001$). However, the model including temperature and pollen origin together was superior to the temperature only model (Table 1b, $P<0.05$) and the null model (Table 1, $P<0.001$). Pairwise comparisons of r (rise) among treatments from the temperature*pollen origin model showed that extreme heat significantly decreased the rise of pollen tube survival relative to control plants and also diminished the differences between cross and self-pollination (Table 2, Figure 2).

Extreme heat reduced the amount of pollen produced per flower by 20% compared to those not exposed to heat stress (Figure S2, Table S1, $P<0.001$).

Extreme heat also significantly reduced seed set (Figure 3, Table S1, $P<0.001$,), and this effect did not depend on pollen origin (Table S1, temperature treatment x pollen origin interaction $P=0.45$).

Discussion

Our results demonstrate that extreme heat can strongly increase the risk of pollen limitation through effects on pollen quality and quantity, which operate at multiple stages of the pollination process (pollen production, pollen tube survival, and seed set). As we predicted, extreme heat decreased pollen quality by diminishing the number of deposited grains that successfully reached the base of the style. Unexpectedly, the effect did not operate in a fully additive way. Instead, the effect of pollen origin on pollen quality was only seen in control conditions. Under extreme heat, the quality of outcrossed and self grains was indistinguishable, albeit much lower than under control conditions. Flowers that developed during extreme heat also produced less pollen compared to control plants. Although we did not measure pollen dispersal via pollinators directly in this study, these reductions are expected to increase the risk of pollen quality and quantity limitation, as supported by the strong negative effects of heat on seed set.

Influences of extreme heat on pollen quality

Extreme heat has been found to affect pollen quality through decreased pollen vigor across multiple taxa (Aloni *et al.* 2001; Young *et al.* 2004; Jiang *et al.* 2019). Our hand pollination treatments support these assessments as heat effects on pollen quality were evident via significant differences in the rise estimates between pollen deposition and pollen tubes (*r*). In flowers that developed during heat treatments, the number of pollen tubes successfully reaching the base of the style increased more gradually over a range of grains deposited. Pollen origin has a well-documented effect on pollen quality (Dogterom *et al.* 2000, Ramsey and Vaughton 2000, Aizen and Harder 2007; Harder and Aizen 2010; Harder, Aizen, Richards, *et al.* 2016) but in our study, somewhat surprisingly, extreme heat nullified the benefits of cross-pollination in comparison to self-pollination. This implies that pollen origin may be less relevant to seed production during heat waves in self-compatible plant species. Indeed, for total seed production and fertilization, pollen dispersal within (self-pollination) and among (cross-pollination) plants should be equally successful so long as pollen grains are dispersed. It is possible that post-pollination effects still might emerge after fertilization (e.g. higher seed abortion, post-fertilization incompatibility), although we did not find any evidence of this from our seed set results.

Influences of extreme heat on pollen quantity

Numerous studies have demonstrated the negative impacts of modest heat on pollen production (e.g. 33C-38C; Prasad *et al.* 1999, 2002; Hedhly *et al.* 2009; Hedhly 2011), which contributes to quantitative pollen limitation. When less pollen is available, stigmas of receptive flowers should receive less pollen even when pollinator visitation is not limited (Cresswell et al. 1999). There may be thermal conditions that increase pollinator activity and elevate visitation in ways that offset negative effects of pollen reduction, although when temperatures are too high these benefits likely diminish (Walters *et al.* 2022; Hemberger *et al.* 2023). In our study, the number of pollen grains produced per flower decreased by 20% in plants exposed to 3 days of heat stress. We made a concerted effort using our hand pollinations to create a full range of pollen deposition among flowers to effectively characterize the relationship between deposition and pollen tube survival. Despite this effort we were unable to achieve high pollen deposition in heat treated plants to match that of control plants (Figure 2). This pattern was likely a function of reduced pollen production. We acknowledge that it could also been due to changes in stigma receptivity and pollen adherence to stigmatic surface (Hedhly *et al.* 2009), which we did not test. However, when considered alongside the depressed pollen production seen in our heat-treated flowers, our hypothesis that decreased pollen production decreased deposition and increased the risk of quantitative pollen limitation seems most likely.

Temporal effects of heat waves on pollen limitation

How dramatically the reduction in pollen quantity and quality limits ovule fertilization and seed production likely depends on the timing of the heat wave relative to flowering. For example, when plants have relatively fewer of their flowers developing or open, heat impacts on pollen limitation should be lower than when they have more developing flowers and more open flowers. Thus, heat waves coinciding with peak flowering will likely have larger fitness impacts than they would for plants at early or late flowering stages. However, it is less clear how heat stress earlier in flowering carries into later flowering periods. Previous work, by Young *et al.* (2004) showed that after a period of recovery, normal floral development occurs again and heat effects on pollen quality and production diminish. The lagged effects of heat waves due to their impact during flower development are nevertheless important for understanding temporal heat wave impacts on pollen limitation at the population level. As flowers that developed during extreme heat open, they contribute relatively less pollen to the pollen pool and increase the risk of quantitative pollen limitation. The longer a heat wave persists the greater the proportion of open flowers within the population are those that experienced heat stress during development. The result is that the daily impact of heat waves will be greater as heat wave duration increases and potential for pollen limitation is magnified. However, da Cunha and Aizen (2023) recently showed plants at peak flowering (larger floral displays) produce more pollen per ovule compared to plants at the beginning and end of flowering (smaller display sizes). If this holds true for plants that develop during heat at peak flowering, then increased pollen production at peak flowering may partially mitigate the impacts of extreme heat on pollen limitation. Future studies should evaluate a large range of heat wave duration, to fully understand expectations of how extreme heat influences the dynamics of pollen quantity and quality limitation. Our short and moderate heat wave (3 days at 35C) had large effects on pollination, and our conceptual framework creates a useful approach for understanding how extreme heat waves will influence pollen limitation in flowering plants.

Implications for different mating systems and life histories

Our research evaluated a single plant species, *Brassica napus*, with a self-compatible mating system. Species with different mating systems may be differentially affected by decreased pollen production and pollen quality caused by extreme heat. Our results suggest that *B. napus* and other species that use self-compatibility for reproductive assurance may not be able to overcome pollen limitation when experiencing the combined negative effects of extreme heat on pollen production and quality. Autogamously pollinated species (those that obligately self) may be even more vulnerable because their flowers produce substantially less pollen compared to plant species that depend on pollen vectors (Harder and Johnson 2023) and this reduction cannot be offset by receipt of outcrossed pollen. Self-incompatible species face a different challenge. Although they produce relatively more pollen, they already generally experience higher rates of pollen limitation relative to those with self-compatible and autogamous mating because they require pollen vectors (either biotic or abiotic) and experience pollen loss during transport (Ashman et al. 2004). For plant species that depend on animal vectors, pollen limitation during heat waves may be additionally increased by changes in pollinator visitation during elevated temperatures; indeed such indirect effects through pollinators may be even larger than direct effects operating on flowers themselves (Walters *et al.* 2022; Hemberger *et al.* 2023; Karban *et al.* 2023). Although our study of a single plant and mating system cannot represent of all possible mating systems, based on studies from different plant species across families, there is a broad congruence of the negative effect of heat stress on flowers across flowering plant families (e.g. Faba beans- Fabaceae: Bishop *et al.* 2016, Rice - Poaeceae: Shi *et al.* 2018, Citrus - Rutaceae: Distefano *et al.* 2018, Cherry - Rosaceae: Hedhly *et al.* 2005).

Consequences of heat-induced pollen limitation for populations

As the frequency, intensity, and duration of heat waves increase (Meehl and Tebaldi 2004), plants that flower during periods of extreme heat are likely to experience profound pollen limitation. Such changes may influence the dynamics of seed-limited plant populations and their capacity to respond to changing climates (Turnbull *et al.* 2000; Ward and Johnson 2005). Protracted heat waves may encompass the entire duration of flowering for some species and limit overall seed set, potentially limiting recruitment and the replenishing of soil seed banks. The extent of this impact will depend on both the species' thermal tolerance and life history characteristics (e.g. generation time, perenniality, seed dormancy). Should heat waves occur across consecutive flowering periods, heat-induced pollen limitation may contribute to seed limitation and decrease local vital rates. How precisely protracted and frequent heat affects plant population demographics is a major knowledge gap and warrants urgent research. In addition to demographic impacts, heat depressed seed production will likely impact how plant populations behave at their range edge (Jump and Peñuelas 2005).

For species whose distributions are shifting, extreme heat may compromise their dispersal and performance at the edges of their range. At the trailing edge of a species range where populations are more vulnerable to extreme heat (see McDonald *et al.* 2023; Holzmann *et al.* 2023), heat-driven pollen limitation and depressed seed production may limit local persistence. In contrast, at the leading edge of a species range, extreme heat wave events may still possible. In this case heat-driven pollen limitation and subsequent bottlenecks in seed production could impede dispersal and migration into more favorable climate envelopes (Chuang and Peterson 2016).

Consequences of extreme heat for pollen limitation in agriculture

The effects of extreme heat on pollen limitation also have important implications for fruit producing agricultural systems, and the stability of agricultural yields. Recent profound heat waves have overlapped with the flowering periods of important agricultural crops in different world regions with devastating effects for yield (Bal *et al.* 2022, White *et al.* 2023). The role of heat-induced pollen limitation was not specifically explored in these cases, but our results suggest it is reasonable that pollen limitation contributed. Although failure in reproduction from heat has not been integrated into the pollen limitation concept previously, a unanimous sensitivity of reproductive organs is seen across plant taxa and growing regions in crop plants. In temperate and subtropical crops show sensitivity to temperatures above 30C-35C (Hedhly 2011). In contrast, these common temperature ranges are normal in the tropics and represent the optima for tropical plants and their flowers. Although warmer, tropical climates tend to be more thermally stable and thus historically have not experienced shortfalls in yield from heat during flowering. However, recent studies suggest that tropical plants are already living close to their thermal maxima (Sentinella *et al.* 2020; Doughty *et al.* 2023). These two factors indicate that tropical plants may be especially sensitive to heat waves should they occur in tropical regions and pollen limitation due to heat may play an important role (Bal *et al.* 2022). We demonstrated in our study that the reproductive organs of a cool adapted crop (*B. napus*) are highly sensitive to relatively modest heat (35C: see also Young *et al*. 2004). Similar impacts have been demonstrated in other cool adapted species, although not for crops, where reproductive organs are sensitive at much lower temperatures (26C: McKee and Richards 1998)**.** Compared to our work, it seems that crop origin and climatic history (mean temperatures at flowering and temperature variance) could be useful for understanding the temperature ranges at which different crops should be vulnerable to pollen limitation. Future research should explore how the origin and historical climate of a crop species influences their sensitivity to high temperatures.

Conclusion

Our work demonstrated how extreme heat decreases pollen availability and quality, which compounds to induce pollen limitation and limit reproductive success in *B. napus*, and this pattern was not altered by pollen origin. *Brassica napus* is highly sensitive to extreme temperatures, and while it may seemingly serve as an extreme response, evidence across different plant species all indicate sensitivity of flowers to heat stress (Hedhly *et al.* 2009, Zinn *et al.* 2010). Therefore, the risk of pollen limitation during periods of extreme heat are likely widespread. Given current and future patterns of extreme heat waves, it is not unreasonable to expect that pollen limitation as a consequence of extreme heat is likely to affect pollination systems globally, potentially bearing negative consequences for the demographics of plants and the stability of agricultural yields and food security.

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Model	df	AIC	Log Likelihood
Null Model (pollen tubes as a function of deposition)	3	1181.06	-587.53
Temperature Treatment Only	4	1164.42	-578.21
Temperature Treatment and Pollen Origin	6	1160.56	-574.28

Table 1a - Model fit and estimates for non-linear pollen tube survival models.

Models compared	Likelihood-ratio	Estimated P values
Null vs. Temperature Treatment Only	18.64	<0.001
Null vs. Temperature Treatment * Pollen Origin	26.49	<0.001
Temperature Treatment * Pollen Origin vs. Temperature Treatment	7.85	<0.05

Table 1b - Results of likelihood ratio tests comparing alternative pollen-tube survival models.

Model	Pairwise Comparison	T ratio	*P* value
r - Number of surviving pollen tubes	heat * self vs. control * self	-3.72	*P*<0.01
	heat * self vs. heat * cross	-0.88	*P*=0.82
	heat * self vs. control * cross	-5.25	*P*<0.01
	control * self vs. heat * cross	3.27	*P*<0.01
	control * self vs. control * cross	-2.84	*P*<0.05
	heat * cross vs. control * cross	-4.83	*P*<0.01
Seed set	heat * self vs. control * self	-6.84	*P*<0.01
	heat * self vs. heat * cross	-0.76	*P*=0.87
	heat * self vs. control * cross	-6.75	*P*<0.01
	control * self vs. heat * cross	6.23	*P*<0.01
	control * self vs. control * cross	0.14	*P*=0.99
	heat * cross vs. control * cross	-6.15	*P*<0.01

Note: Number of surviving pollen tubes tests pairwise comparisons between model estimates of *r* (see Equation 1). Seed set represents results of pairwise comparison between marginal means in seed set model.

Table 2 **-** Pairwise comparisons of temperature * pollen origin treatments for *r* parameters and marginal means for the pollen tube survival and seed set, respectively. In the pairwise comparison column the level of the interaction indicated and which level it is being compared to.

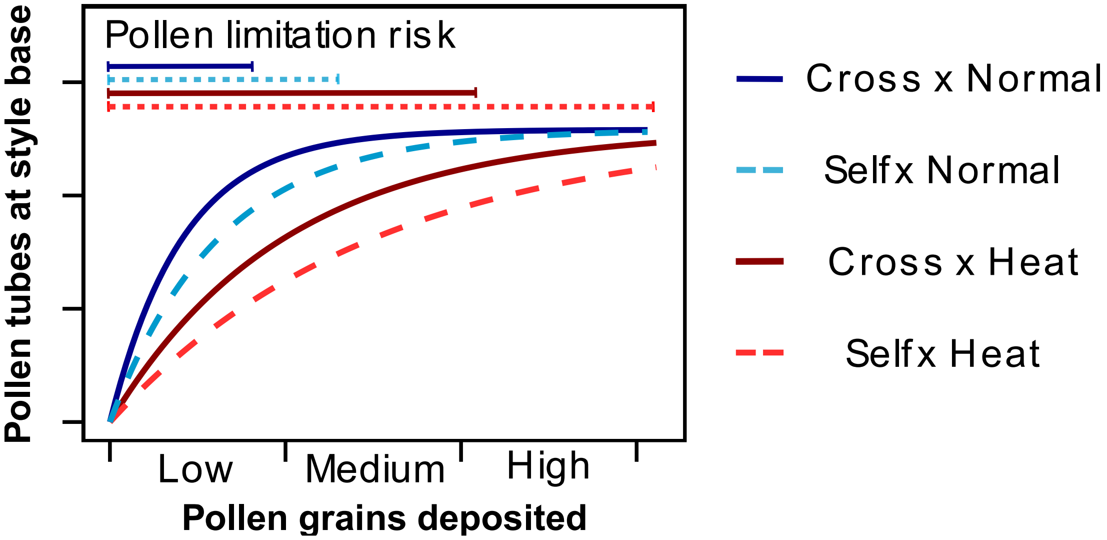

Figure 1: - Represents the relationship between the number of pollen grains deposited and the number of pollen tubes surviving to the base of the style which may be able to fertilize ovules. Colour represents the conditions of pollen during pollen development (blue - normal temperatures, red - extreme heat). Line type represents the origin of pollen (solid - outcross, dashed-self). Barred lines represent the range of pollen deposition that leads to a risk in pollen limitation. Bins on the x axis indicate regions qualitatively considered low, medium, and high pollen receipt.

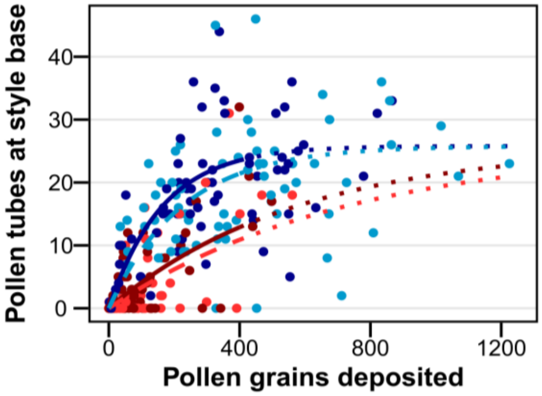

Figure 2 - Model predicted values between pollen tubes reaching the base of the style and the number of pollen grains deposited. Trend lines are estimated from a model subset to <400 pollen grains which utilizes a common fixed asymptote estimated for control and cross-pollinated plants in the full model. Solid lines represent cross-pollination and dashed lines represent self-pollination. Blue represents control plants (dark blue = outcross, light blue = self), whereas red represents heat treatments (dark red = outcross, light red = self). Dotted lines continuing represent extrapolation of the model not fit to data.

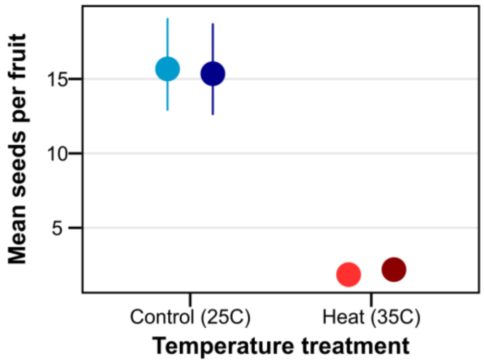

Figure 3 – Estimated marginal mean number of seeds per fruit (± SE) from temperature and pollen origin treatments. Colour represents pollen origin treatment (light blue = control*self, dark blue = control*outcross, light red = heat*self, dark red = heat*outcross).

Supporting Information

Model	Link function	Parameter	Estimate ± SE	Z value	P value
Pollen tube survival	None	*r* - Heat / Self	1.37x10^-2±5.70x10^-3		
		r - Control / Self	4.43x10^-2±5.94x10^-3		
		r - Heat / Cross	1.73x10^-2±5.74x10^-3		
		r - Control / Cross	6.05x10^-2±6.86x10^-3		
Pollen production	None	Intercept	17328±1146	15.12	P<0.01
		Plant treatment	4452±1605	2.77	P<0.01
Seed set	log	Intercept	0.62±0.24	2.55	P<0.05
		Temperature treatment	2.13±0.31	6.84	P<0.01
		Pollen origin treatment	0.17±0.22	0.76	0.45
		Temperature - Pollen Origin Interaction	-0.19±0.26	-0.71	0.48

Table S1 - Parameter estimates from pollen tube survival models, pollen production, and seed set. The pollen tube model does not include significance values as tests from these analyses are reported in Tables 1 and 2.

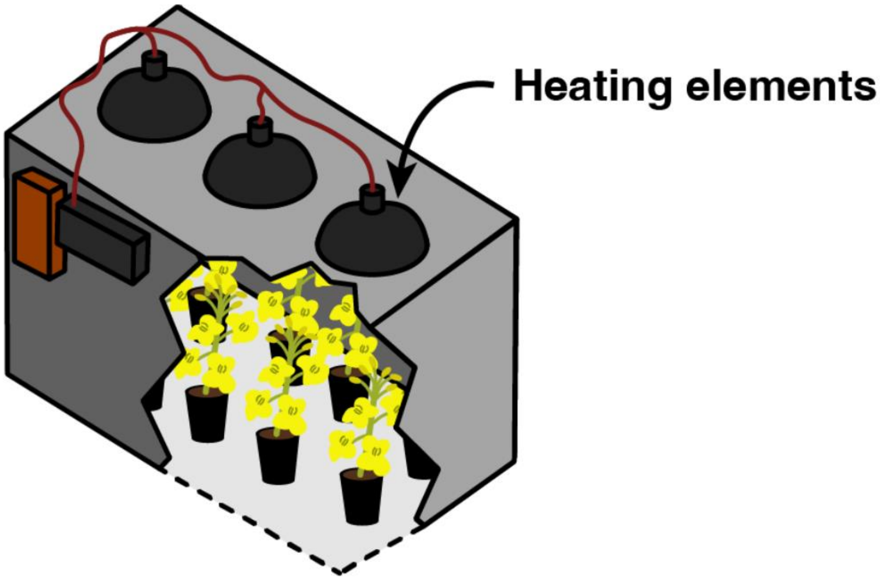

Figure S1 – Diagram shows the growth chamber used to experimentally treat plants with heat. Ceramic heating elements attached to the top of the chamber regulated temperature via attached thermometer and computer.

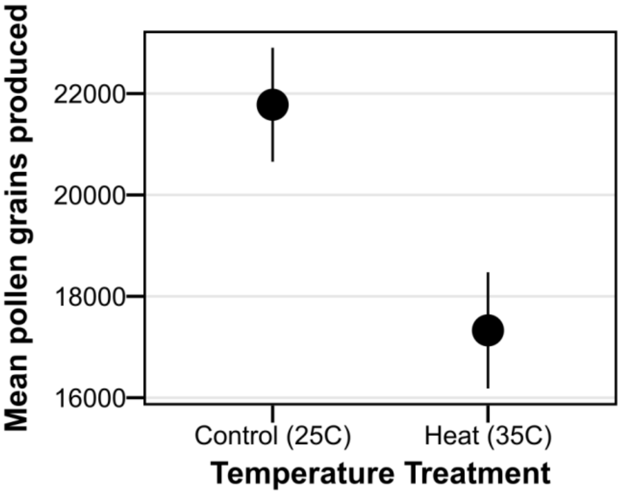

Figure S2 - Mean number of pollen grains produced by a flower in response to temperature treatment ± SE. Means are estimated marginal means.

Chapter 2: Experimental heat waves decrease pollination and plant reproductive success through direct and indirect effects on flowers and pollinators

Introduction

Climate instability driven by global heating is increasing the frequency, duration and intensity of extreme heat events (i.e. heat waves; Meehl and Tebaldi 2004; Stillman 2019; Thiery *et al.* 2021), which poses a serious threat to the persistence and fitness of organisms that experience them (Kingsolver *et al.* 2013; Harvey *et al.* 2023). Extreme heat threatens organisms when temperatures exceed an organism's thermal maxima which can cause mortality (Deutsch *et al.* 2008; Clusella-Trullas *et al.* 2011; Vasseur *et al.* 2014), disrupt reproduction (Hedhly *et al.* 2009; Hansen 2009; Hedhly 2011) and essential ecological interactions that span crucial life history periods (Harvey et al. 2022, Johnson et al. 2023). One essential ecological interaction and ecosystem service that may be highly sensitive to extreme heat is animal-mediated pollination (Bishop et al. 2016, Hemberger et al. 2022, Walters et al. 2022, Potts et al. 2016). Extreme heat may impact animal-mediated pollination directly through heat effects on flowers and their reproductive organs (Hedhly *et al.* 2009; Hedhly 2011; Distefano *et al.* 2018), and also indirectly through effects on foraging pollinators (Walters *et al.* 2022; Hemberger *et al.* 2023). Although some studies have explored how extreme heat impacts flowers (Bishop *et al.* 2016) and pollinators individually (Kenna *et al.* 2021; Gérard *et al.* 2022) we have limited understanding, and no empirical evidence of the absolute and relative importance of extreme heat's direct and indirect effects on the dynamics of animal-mediated pollination and floral reproduction.

Extreme heat can directly influence the dynamics of pollination and reproduction by compromising flowers and their reproductive organs during floral development and bloom

(Hedhly *et al.* 2009; Hedhly 2011; Bishop *et al.* 2016; Distefano *et al.* 2018; Chapter 1), and by altering plant attractiveness through floral rewards (Descamps *et al.* 2021; Hemberger *et al.* 2023). Flowers that develop in extreme heat produce fewer, less viable pollen grains (Young *et al.* 2004; Hedhly *et al.* 2009; Zinn *et al.* 2010). Fewer pollen grains produced per flower reduces the pool of pollen that can be dispersed to receptive stigmas, which consequentially decreases pollen export and deposition by visiting pollinators (Cresswell 1999). Of those pollen grains that pollinators do deposit, lower pollen viability reduces their post-pollination success and reduces the likelihood that each deposited pollen grain produces a pollen tube and that its pollen tube reaches the base of the style to enter the ovary. In addition to these effects of extreme heat on gametes, extreme heat also reduces nectar production (Hemberger *et al.* 2022). Reduced nectar and pollen production from extreme heat's direct effects on flowers can inadvertently decrease flower attractiveness to visiting pollinators (Descamps *et al.* 2021), which may further reduce pollen export and deposition. Although extreme heat's direct effects on pollination through floral development may be profound, its indirect effect on foraging pollinator may also be limiting.

Extreme heat may indirectly limit pollination by increasing the energetic costs and needs of foraging pollinators (Kenna *et al.* 2021; Harvey *et al.* 2023) and altering cognition (Gérard *et al.* 2022), which alters their foraging behavior and ability to mediate pollination (Walters *et al.* 2022; Hemberger *et al.* 2023,). When pollinators forage in extreme heat they expend more energy to thermoregulate and meet elevated metabolic needs. This energy expenditure requires pollinators to either increase resource intake or limit their activity to conserve energy. Given that extreme heat also reduces the pollen and nectar rewards of individual flowers (Hemberger *et al.* 2023), pollinators deplete floral rewards more quickly and spend less time foraging (Hemberger *et al.* 2023). Furthermore, even short exposure to extreme heat can decrease pollinator cognition and their ability to handle and recognize flowers (Gérard *et al.* 2022). Together, these impacts of extreme heat on a pollinator fitness and foraging behavior decreases the number of plants and flowers that pollinator visit (Hemberger *et al.* 2023), which likely decreases pollen export and deposition. Understanding heat's indirect effects on pollination through foraging pollinators requires understanding its context relative to direct effects on flowers throughout the pollination, post-pollination and reproductive processes.

Direct and indirect impacts of extreme heat on developing flowers and foraging pollinators, respectively, likely limit reproduction through successive bottlenecks during the pollination and post-pollination processes. Extreme heat may bottleneck reproduction initially via direct and indirect effects at the pollen dispersal stage through reduced pollen production and fewer pollinator visits. Following the success of these pollen grains, extreme heat could secondarily bottleneck reproduction post-pollination through reduced pollen grain success and pollen tubes reaching the base of the style and ovary. Together these components determine to what degree reproduction is limited by pollen receipt (i.e. pollen limitation; Aizen and Harder 2007). Partitioning the absolute and relative contributions of extreme heat's direct and indirect impacts to pollination is essential to understand how wild ecosystems and agricultural systems will respond to extreme heat wave events. To our knowledge no prior study has partitioned the absolute and relative effect of extreme heat's direct and indirect effects on pollination and floral reproduction. Importantly, extreme heat's direct and indirect effects on pollination likely to occur independently and concurrently over a heat wave's temporal progression.

The relative dominance of extreme heats' direct and indirect effects on pollination and reproduction should transition over a heat wave when developing flowers and foraging pollinators experience heat. Hypothetically, at heat wave onset pollinators cope with high temperatures during foraging (i.e. reduced visitation), whereas open receptive flowers developed during normal temperatures (normal pollen production per flower and viability) and thus heat limits pollination indirectly (Figure 1). As the heat wave continues and until cessation, flowers that developed in extreme heat become receptive and pollinators forage, which reduces pollination directly and indirectly. Immediately after the heat wave stops, alleviated pollinators forage in normal temperatures, but receptive flowers developed during extreme heat. Therefore, direct effects should limit pollination and reproduction post-heat wave until plants recover from heat's direct effects.

We designed a two factor, fully-crossed experiment where we exposed plants during floral development and pollinators during foraging to extreme heat so that we could partition the relative and combined contributions of extreme heat's direct and indirect effects on pollination and reproduction. We measured pollination (pollen removal and pollen deposition), post-pollination (pollen viability, pollen tube survival), and reproduction (fruit set, and seed set) to evaluate ultimate reproductive impacts of extreme heat throughout each stage of the mating process. Evaluating each stage is essential to disentangle the relative importance of each mechanism and what ultimately constrains successful reproduction. In addition to this detailed partitioning, our experiment informs how direct and indirect effects drive pollination and reproduction throughout a heat wave. Specifically, we predict that in response to extreme heat:

1. Pollen removal and deposition (pollination) decreases from direct heat effects during flower development and deposited pollen grains have lower viability and pollen tube success (post-pollination) which together strongly limit fruit set and seed set (reproduction) (Figure 1).

2. Pollen removal and deposition (pollination) decreases from indirect effects as pollinators visit fewer flowers. Of pollen grains that are deposited pollen grains have normal viability and pollen tube success (post-pollination) and therefore fruit set and seed set (reproduction) are limited by pollination (Figure 1).
3. Pollen removal and deposition (pollination) decreases from combined direct and indirect effects during floral development and pollinator foraging. Deposited pollen grains have lower viability and pollen tube success (post-pollination from direct effects) and therefore both pollination and post-pollination limit fruit set and seed set (reproduction: Figure 1).

Methods

Experimental design

To quantify the direct and indirect effects of heat waves on plant-pollinator interactions we designed a fully-crossed 2 x 2 experiment that manipulated flower development temperature, and pollinator foraging temperature (Figure 1; Hemberger et al 2022). The first treatment (control) represents our expectations of pollination under normal conditions when flowers develop and pollinators forage at ambient temperature (25C: for both flower development and pollinator foraging). The second treatment represents conditions at the onset of a heat wave when flowers have developed at normal temperatures, but pollinators forage under extreme heat (25C: for flower development, 35C: for pollinator foraging). The third treatment represents a heat wave peak where flowers develop in extreme heat and pollinators forage in extreme heat (35C: for flower development and pollinator foraging). The fourth treatment represents plant-pollinator interactions immediately after the heat wave subsides where open flowers have developed during extreme heat, but pollinators are at last freed from extreme heat (35C: for flower development, and 25C: for pollinator foraging).

Study system

We conducted our experiments using the plant *Brassica napus* (i.e. rapeseed or Canola) and the bumble bee pollinator *Bombus impatiens* (i.e. Common eastern bumble bee). *Brassica napus* is a globally-distributed temperate adapted crop (grown between ~30N-60N Northern hemisphere, and ~30S-60S southern hemisphere) which is sensitive to heat during flowering, and during seed development (Young *et al.* 2004). *Bombus impatiens* is the dominant wild pollinator of both native plant communities and multiple crops in eastern North America. We chose 35C to represent the conditions of a heat wave as this temperature represents a reasonable heat wave within the growing region *B. napus* (Young *et al.* 2004), and range of *B. impatiens*.

Experimental heat treatments: Plants

For our experiments we used a rapid cycling *B. napus* variety (Rapid Cycling Brassica Collection (RCBC), University of Wisconsin - Madison). We seeded 5cmx5cm pots with *B. napus* to produce cohorts of 60 plants in February and March 2021 at the University of California - Davis. Every 3 days we repeated this to create a constant supply of plants. We grew seedlings inside under constant full spectrum light until they developed their first true leaves, after which we moved them into a greenhouse for rearing. Within the greenhouse we reared plants at 25C under a 16hr/8hr day:night and watered them daily. We additionally fertilized plants every 3 days with Peters Professional Fertilizer (ICL Growing Solutions) with micronutrients at 1:1:1, N:P:K at 100 ppm volume to prevent resource limitation and promote healthy plant growth. When individual plants began to flower (approximately 6 weeks after seeding) and develop branching racemes we moved them into treatment chambers for experimental heating.

From each cohort we placed 9 plants in an experimental heating chamber and 9 plants into a replicate control chamber (Figure S1). Plants were kept in their respective treatment chambers for 72 hours and under the same 16hr/8hr day:night cycle with full spectrum grow lights. In the heating chamber, ceramic heat lamps were used to elevate and regulate daytime temperatures to ~35C to simulate heat wave conditions. In the control chamber, we treated plants at 25C to simulate normal temperature conditions. The heating elements were turned off during the night to simulate nighttime cooling. After this 72 hour exposure, we removed branching racemes so that each plant had approximately 9 flowers to hold display size constant - (9.33 mean flowers +-0.11SE)). Then we placed the 9 treated plants into the foraging chamber and positioned them in a 3x3 array.

Experimental heat treatments: Bumble bees

We established bumble bee micro-colonies which are commonly used in experimental settings (e.g., Dance *et al.* 2017; Hemberger *et al.* 2020). Micro-colonies are small colonies consisting solely of workers and without a queen. We haphazardly selected 5 bumblebee workers from one of five source colonies (BioBest Biological, Romulus, Michigan). We started microcolonies with ~2g pollen balls, and abundant nectar. We established two micro-colonies 10 days prior to experimental foraging trials and continued establishing two colonies daily for 20 days to total 40 micro-colonies, so that a single micro colony could be used in each trial. Using this establishment regime allowed us to standardize micro-colony age for the experimental foraging trials. Once established, we fed bumble bees every 2 days with ample pollen and nectar. This micro-colony development period was essential to initiate normal colony behaviors (i.e. brood maintenance, and foraging) after egg laying and larval development. We kept microcolonies in a dark room at 25C throughout micro-colony development. Previous work has shown that bumble bee circadian rhythm is consistent in the absence of light (Stelzer and Chittka 2010; Tasman *et al.* 2020), and thus micro-colony development should be unaffected. Two days prior to the foraging trial, we attached microcolonies to training chambers containing multiple dwarf *B. napus* plants to familiarize colonies flowers and their rewards along with diurnal periods.

We constructed two 120cmx120cmx120cm foraging chambers built with rigid styrofoam fixed to a metal frame (Figure S1). For one chamber we held the temperature constant at 25C as a control chamber and the second at 35C as a heat treatment chamber. We used temperature logger to record and regulate temperature within each chamber. In each chamber we placed 9 plants as in a 3x3 array where plants had gone through 3 days of temperature treatment (25C - control and 35C - heat). We then attached the *B. napus* trained microcolonies to each chamber to allow them the freedom to forage for nectar and pollen and visit flowers for a 6 hour foraging window (Figure S1). We set up a raspberry pi microcomputer with an attached camera to record bumble bee behavior, and flower visits (reported in Hemberger *et al.* 2023). We repeated each treatment 10 times, for a total of 40 trials. Therefore 90 individual plants were used per treatment – totaling 360 plants across all treatments.

Pollen removal

To measure pollen removal, we carefully collected anthers from 3 flowers of each plant immediately after foraging trials and then returned the plants to the greenhouse. We sampled anthers from three different positions on each plant (bottom, middle, or top of the inflorescence) to ensure we represented a range of flower ages in our estimates. We placed anthers from flowers in 70% Ethanol inside 1.5 mL Eppendorf tubes for later processing and counting. Importantly, we recognize that flowers that opened at the heat wave onset had already developed pollen grains, and so may not perfectly represent pollen production, but instead availability.

We used a Coulter Multisizer 3 (Beckman Coulter) to count pollen grains from anthers that had been stored in 70% ethanol. First, we vortexed Eppendorf tubes containing anthers, transferred their contents into a cuvette and sonicated them to release any residual pollen. Second, we rinsed individual anthers after sonication with saline solution to ensure all pollen remained in the cuvette (0.9% NaCl). Before sampling with the Multisizer, we gently rotated cuvettes to agitate pollen grains and disperse them equally throughout the solution. Third, we weighed the filled weight of the cuvette to calculate total volume before sampling with the particle counter. Finally, we counted the number of particles in 1 mL of solution between 20-30 microns. This process was repeated two additional times with each sample for a total of three measurements. Particles between the size of 20-30 microns were considered pollen grains as the particle peak always fell within this range, and we validated pollen ID and size by measuring samples of grains under the microscope. We took the average of these 3 samples and multiplied it by the volume in ml to calculate the total number of pollen grains in each sample. This value represents is the number of pollen grains remaining per flower.

Pollen deposition and pollen tubes

To measure pollen deposition, individual pollen grain success, and pollen tube survival, we collected styles from the same 3 flowers used to measure pollen removal 24 hours after the trial ended to ensure sufficient pollen tube growth (Young *et al.* 2004). We then stored the collected styles in 70% ethanol for later staining. We prepared styles for staining by initially rinsing with DI water and then soaking in DI water for 1 hour. We transferred rinsed styles to a 8M NaOH solution and heated them on a hot plate for 1 hour at 55C to soften style tissues. We then rinsed styles again with DI water and repeated the 1 hour DI water soak before moving them to 0.1M K_3PO_4 buffer with 1% aniline blue dye to stain (mixed at 90% K_3PO_4 solution and 10% aniline blue dye solution). We then kept styles in the refrigerator for 24 hours before mounting and squashing them on microscope slides for observation under a fluorescent microscope. We fluoresced styles under UV light and counted the number of pollen grains on the stigma and the number of pollen tubes that survived to the base of the style (Martin 1959). These measurements together inform at the individual flower level how much pollen was deposited during experimental trials, and how those pollen grains responded to the treatments post-pollination.

Plant reproduction: Fruit set and seed set

We kept trialed plants within the greenhouse and continued their fertilizer regime for an additional 20 days after pollination before exclusively watering until flower production stopped. We collected fruits at maturation from target flowers to measure fruit set and seed set. We determined fruit set based on ovary enlargement at maturation. Once fruits were mature and yellowing, we collected them and placed them into coin envelopes to ensure desiccation and prevent mold growth. We collected all ovaries from which we had also collected styles. We removed the fruits from coin envelopes in the lab and counted the number of seeds produced in each fruit.

Data Analysis

We analyzed all data using the software program R (v4.3.1) and constructed generalized linear mixed models using the package “glmmTMB” for all analyses (Brooks *et al.* 2023). We considered the dependent variables in our models: pollen grains remaining per flower, pollen grains deposited per stigma, probability of pollen grain success (i.e. pollen viability), the number of pollen tubes at base of the style (i.e. pollen tube survival), fruit set and number of seeds per fruit (i.e. seed set). Each measurement came from an individual flower which was replicated across 3 flowers from each plant, from every plant, per trial. Therefore, we included foraging trial and plant ID as random effects, with plant ID nested within trial. Our independent variables involved flower development temperature treatment and bumble bee foraging temperature treatment as well as their interaction. Significant effects from floral development indicate the direct effect of extreme heat on pollination at the flower level whereas significant effects of bee foraging temperature indicate the indirect effect of extreme heat on pollination. Models assumed appropriate error distributions congruent with the nature of the data. For continuous models pollen remaining per flower we used a normal distribution (see transformation above). For discrete variable based models (pollen deposition per flower, pollen tube survival, and seed set) we considered both Poisson and negative binomial distributions. For our binary variables (probability of pollen grain success and fruit set) we considered binomial or beta-binomial distributions. To clarify, deposited pollen grain success involved a Bernoulli process and therefore we considered the number of pollen grains deposited on a stigma as the number of trials, and the number of pollen tubes as the base of the style from that sample as successes. Thus, the output from this model tells us what the probability is that an individual deposited pollen grain produces a pollen tube that reaches the base of the style. We selected the response variable distributions using Aikake's Information Criterion as the final analysis. From each model we analyzed Wald type III chi-squared tests and produced estimated marginal means using the package "emmeans" (Lenth *et al.* 2023).

Results

From our experimental trials we found extreme heat had strong negative effects through both direct effects of flower development and indirect effects through bumble bee foraging on pollination, post-pollination, and reproduction.

Pollination

Extreme heat did not affect pollen removal, but it did dramatically affect pollen deposition through direct and indirect effects on flower development and bumble bee foraging. Extreme heat did not reduce pollen remaining per flower through either the direct effect of heat on flower development ($\chi2 = 1.55$, $P=0.12$, Table 1, Figure 2A) or through indirect effects on bumble bee foraging ($\chi2 =-0.35$, $P=0.72$, Table 1), and pollen remaining from flower development did not depend on bumble bee foraging treatment ($\chi2 =-0.95$, $P=0.37$, Table 1). Extreme heat reduced pollen deposition through direct effects on floral development ($\chi2 =3.16$, $P<0.01$, Table 1, Figure 2B) and bumble bee foraging ($\chi2 =1.99$, $P<0.05$, Table 1), but flower development treatment did not depend on bumble bee foraging ($\chi2 =0.10$, $P=0.92$, Table 1).

Extreme heat reduced pollen deposition directly through flower development by 71% and decreased indirectly through bumble bee foraging by 55% (Figure 2B), whereas concurrent extreme heat decreased pollen deposition by 87%.

Post-pollination

Extreme heat also negatively affected post-pollination processes via direct and indirect pathways. Extreme heat dramatically decreased probability of individual deposited pollen grain success (pollen viability) directly through flower development ($\chi2=3.32$, $P<0.001$, Table 1, Figure 2C), but not through bumble bee foraging ($\chi2=-0.27$, $P=0.79$, Table 1). Nor did the effect of extreme heat on individual pollen grain success through floral development depend on bumble bee foraging treatment. Deposited pollen grains from flowers that had developed during extreme heat were 80% less successful than those that developed at control temperatures. The number of pollen tubes reaching the base of the style matched patterns of pollen deposition, such that extreme heat had direct negative effects on pollen tube survival through flower development ($\chi2=5.10$, $P<0.001$, Table 1, Figure 2D) and indirect negative effects through bumble bee foraging ($\chi2=2.82$, $P<0.01$, Table 1). However, extreme heats effect on the number of pollen tubes at the style base through flower development did not depend on whether bumble bees foraged in extreme heat ($\chi2=-0.79$, $P=0.428$, Table 1). Extreme heat reduced pollen tubes reaching the styled directly through flower development by approximately 95%, and indirectly through bumble bee foraging by 73%. When extreme heat was concurrent, pollen tubes reaching the style base declined by 99% compared to control conditions.

Reproduction

Extreme heat dramatically reduced reproduction both directly through flower development and indirectly through bumble bee foraging. Extreme heat significantly decreased fruit set through direct effects only during flower development ($\chi2$=2.04, $P<0.05$, Table 1, Figure 2E), but not through indirect effects on bumble bee foraging. Furthermore, and this direct effect did not depend on bumble bee foraging temperature treatment ($\chi2$=0.33, P=0.74, Table 1). This direct effect of extreme heat on seed set through flower development was marginal and only decreased fruit set by 7%. Extreme heat directly reduced seed set through direct effects on flower development ($\chi2$=6.53, $P<0.001$, Table 1, Figure 2F), and also indirectly reduced seed set through bumble bee foraging ($\chi2$=2.85, $P<0.01$, Table 1). Importantly, this indirect effect did not depend on whether flowers developed during extreme heat ($\chi2$=-1.04, P=0.30, Table 1). Extreme heat reduced seed set directly through floral development by approximately 97%, whereas it indirectly reduced seed set through bumble bee foraging by 67% (Figure 2F). When extreme heat was concurrent, seed set declined by 99%.

Discussion

Extreme heat is increasingly prevalent in a world undergoing rapid climate warming and exposes flowering plants and their pollinators to temperatures that disrupt pollination (Hedhly *et al.* 2009; Bishop *et al.* 2016; Nicholson and Egan 2020; Hemberger *et al.* 2023; Harvey *et al.* 2023). However, studies to date have been primarily limited to only plant responses (Bishop *et al.* 2016), or only pollinator responses to extreme heat (Kenna *et al.* 2021; Gérard *et al.* 2022). While these impacts to pollination have generally been negative when plants are exposed to heat and presumed negative via impacts on pollinators, we found strong negative effects on pollination and reproduction. Furthermore, our work reveals intricate nuances driven by both extreme heat's direct and indirect effects. Specifically, we show that direct and indirect effects of extreme heat additively reduced pollination (pollen deposition direct: 71%, indirect: 55%, both: 87%), but low pollen viability from floral development (direct: 80% lower) further reduced reproduction in flowers that developed during extreme heat regardless of whether bumble bees foraged in extreme heat (seed set, direct: 97%, indirect: 67%, both: 99%). These results demonstrate that although both direct and indirect effects can strongly limit pollination and reproduction, extreme heat's direct effects are dominant. Our results also confirm previous work showing the sensitivity of flowers to heat (Hedhly *et al.* 2009; Hedhly 2011; Bishop *et al.* 2016; Distefano *et al.* 2018) and demonstrate the hypothesized effect of extreme heats influence on pollinators for pollination (Walters *et al.* 2022; Hemberger *et al.* 2023). Furthermore, our experiment integrated a temporal perspective to represent how pollination should change across the progression of a heat wave. Taken together, our results demonstrate that early on in a heat wave, indirect effects of heat on pollinator foraging will be the main driver limiting pollination, before transitioning into a later phase when direct effects of extreme heat on flower development override initial indirect effects by collapsing pollen viability at peak, and post-heat wave.

Direct effects

Extreme heat during floral development imposed strong direct effects on pollination, post-pollination, and reproduction. Floral reproductive structures and gametes are highly sensitive to temperature during development (cite), and extreme heat depresses reproduction during mating among plant and animal taxa (Hedhly 2011; Li *et al.* 2017; Walsh *et al.* 2019; Martínez-De León *et al.* 2023). Although we did not find significant effects of extreme heat on pollen removal, the general patterns observed reflected our predictions. However, the other component of pollination, pollen deposition, was highly sensitive to extreme heat.

Decreased pollen production from extreme heat and decreased plant attractiveness strongly bottlenecked pollen deposition, however direct effects on pollen viability created a strong secondary reproductive bottleneck. Because extreme heat reduced pollen viability by 80%, individual flowers would need to receive 5 times the amount of pollen to set enough pollen tubes to match control conditions. This pollination shortfall could be offset by increased pollinator visitation if pollinators deposit more pollen. However, receiving 5 times the quantity of pollen may lead to stigma clogging (i.e. excess deposited pollen grains block further pollen adhesion: Barrett 2002) or high pollen tube competition which could still limit final pollen tube success (Cruzan 1986; Harder *et al.* 2016). Critically, our results demonstrate that extreme heat limits pollinator visitation (reported in Hemberger *et al.* 2023), which means receiving additional pollinator visits to make up for post-pollination effects would be challenging to overcome. However, how this plays out in more natural systems needs additional study as other more heat tolerant pollinator species may be able to offset these strong direct post-pollination effects (Harvey *et al.* 2020, 2023; Ma *et al.* 2021).

Indirect effects

Although direct effects of extreme heat on reproduction dramatically limited pollination and reproduction, indirect effects of extreme heat on pollinator foraging are also strongly limiting. When bumble bees foraged in extreme heat and flowers developed in normal temperatures, pollen deposition decreased by 55% and reduced seed set by 67%. This was likely due to decreased flower and plant visitation, and shorter foraging periods observed when bumble bees were exposed to extreme heat (results reported in Hemberger *et al.* 2023). During foraging trials, bumble bees spent similar time per flower for each flower visit regardless of foraging treatment. This implies that differences in pollination between treatments were driven by reduced pollen production and reduced total flower visits per bumble bee rather than specific changes in bumble bee behavior. As pollen viability was unaffected by bumble bee foraging temperature, our results and other studies (see Bishop *et al.* 2016) indicate that plants exposed to extreme heat are more dependent on pollinators for successful reproduction. Insects' ability to cope with extreme heat and forage to disperse pollen is highly influenced by their thermal optima, and their body size which dictates how quickly they are able to shed excess heat, as smaller insects heat up and cool down more quickly (Pereboom and Biesmeijer 2003). However, under *in situ* foraging conditions bumble bee pollinators could still disperse pollen in the margins of a daylight period, potentially shifting their foraging to periods earlier in the morning or later in the evening to select for cooler/optimal foraging temperatures (Stelzer *et al*. 2010, Walters *et al.* 2022).

Impacts to plant populations

Our results demonstrate that reproduction is strongly limited by extreme heat’s direct and indirect effects, which implies that they will be disruptive to seed production and successful mating between plants. Given the increasing frequency, duration and intensity of heat waves (Meehl and Talbaldi 2004; Stillman 2019; Thiery *et al.* 2021), direct and indirect effects of extreme heat are likely to limit total plant reproductive output and may disrupt adaptive mating opportunities. If a heat wave completely overlaps with the flowering period of a plant, the combined effects of reduced pollen production, pollen viability and decreased pollinator visitation likely disrupts gross seed output. Reduced seed output from extreme heat may also limit the capacity of plant species to disperse into suitable climate envelopes at the leading edge of a species distribution, and potentially accelerate local extinction at trailing edges (Jump and Peñuelas 2005). From a perspective of selection, extreme heat is likely to strongly select for heat tolerance in flowers during floral development and receptivity. However, this strong selection and disruption of mating could weaken selection by other abiotic (e.g. desiccation resistance, drought tolerance) and biotic pressures (e.g. novel species interactions, herbivory) in individuals whose flowers and gametes are vulnerable to extreme heat (Barrett and Harder 2017).

Impacts to agriculture

Our results suggest that agricultural systems and crop yields will be highly vulnerable to extreme heat during heat waves. We found strong negative effects of extreme heat on the direct and indirect pathways of the crop plant *Brassica napus*. Our evidence of extreme heat's effects pollination and reproduction and underscores the potential for pitfalls in yield crop plants may experience during heat waves. The negative direct effects heat imposes during floral development is widespread across crop plants (Young *et al.* 2004; Hedhly *et al.* 2009; Hedhly 2011; Bishop *et al.* 2016; Distefano *et al.* 2018). These direct effects alone suggest that extreme heat will not only limit plants dependent on animal-pollinators, but also those that employ abiotic vectors. Furthermore, for animal-pollinated plants we find the indirect shortfalls in pollination caused by reduced pollinator visitation (Hemberger *et al.* 2023) alarming because heat stressed plants are more dependent on animal pollinators to overcome pollination shortfalls (Bishop *et al.* 2016). In our experimental design, we kept the size of micro-colonies constant across treatments to 5 bumble bee workers. Hypothetically, increasing the abundance of pollinators could be a way over overcoming the pollination deficit, but we did not test that in our study. Still, given concern about global pollinator declines, this deficit may be difficult to overcome through wild pollinator populations (Potts *et al.* 2010, 2016; Osterman *et al.* 2021), and may put more pressure on growers to rely on managed pollinators during periods of extreme heat.

Heat wave progression

Our experiment assessed conditions that promote extreme heat's direct and indirect effects over the progression of a heat wave. Using our temporal framework established in Figure 1, our results suggest that at the onset of a heat wave, pollinators deliver decreased pollination services, through strong indirect effects. However, as the heat wave enters its peak the combined direct and indirect effects collapse pollination and reproductive success. This implies that mating opportunities and reproduction during a heat wave are marginal at best. The lagged effects from extreme heat on flowers carries over post-heat wave, and because of the effects on pollen production and pollen viability, reproduction is still reduced until plants recover. Although these results are informative, our study still likely underestimates a heat wave's effects on pollination. We designed our experiment to be fully independent between treatments and so we do not capture progressive effects. Likely, at the peak of a heat wave, post heat wave pollinators will be responding to the reduced resource intake over the preceding days (Walters *et al.* 2022; Hemberger *et al.* 2023). Hypothetically in this post-heat wave period, starving pollinators would lethargically forage at flowers and hence export and deposit less pollen than our approximations suggest (Hemberger *et al.* 2023).

Conclusion

Our experimental heat wave profoundly limited pollination and reproduction through extreme heat's direct effects on flower development and indirect effects through pollinator foraging. Importantly, while indirect effects of extreme heat on pollination through pollinator foraging dramatically decreased reproductive output, the direct effects of extreme heat on flower development for pollination and post-pollination dwarfed their indirect effects. As climate conditions warm and become more unstable, extreme heat's direct and indirect impacts on pollination will become more prevalent and disruptive to wild plant mating and reproduction and agricultural crop yields. Although our elegant experiment reveals dramatic impacts of heat, it does not capture all the processes associated with heat waves and extreme heat (e.g. increased water stress). Consideration of multiplicative stressors during flowering would further illuminate our understanding of how animal-mediated pollination will change in response to global heating. In essence, our results underscore the profound threat that climate-amplified heat waves and extreme heat pose to animal-mediated pollination, reproduction in plant communities (Jump and Peñuelas 2005), and to yield stability in fruit bearing crops (Battisti and Naylor 2009; Lesk *et al.* 2016).

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Model	Distribution and *Link Function*	Parameter	Estimate ±SE	Z-value	*P* value
Pollen remaining	Gaussian	Intercept	17461±2402	7.27	**<0.001**
		Bee Treatment	-1192±3380	-0.35	0.72
		Flower Treatment	5183±3354	1.55	0.12
		Bee Treatment x Flower Treatment	-4312±4763	-0.91	0.37
Pollen deposition	Quasi-Poisson - *Log*	Intercept	3.21±0.27	11.74	**<0.001**
		Bee Treatment	0.76±0.38	2	**<0.05**
		Flower Treatment	1.20±0.38	3.16	**<0.01**
		Bee Treatment x Flower Treatment	0.06±0.53	0.1	0.92
Probability of pollen success	Beta-Binomial - *Logit*	Intercept	-4.36±0.41	-10.57	**<0.001**
		Bee Treatment	0.53+-0.55	0.97	0.33
		Flower Treatment	1.75+-0.53	3.32	**<0.001**
		Bee Treatment x Flower Treatment	-0.19±0.71	-0.27	0.79
Pollen tube survival	Negative Binomial - *Log*	Intercept	-2.63±0.56	-4.68	**<0.001**
		Bee Treatment	2.10±0.75	2.82	**<0.01**
		Flower Treatment	3.79±0.74	5.1	**<0.001**
		Bee Treatment x Flower Treatment	-0.80±1.01	-0.79	0.43
Fruit set	Binomial - *Logit*	Intercept	0.70±0.08	9.11	**<0.001**
		Bee Treatment	0.04±0.11	0.34	0.74
		Flower Treatment	0.23±0.12	2.04	**<0.05**
		Bee Treatment x Flower Treatment	0.051±0.16	0.33	0.74
Seed set	Quasi Poisson - *Log*	Intercept	-3.21±0.58	-5.51	**<0.001**
		Bee Treatment	1.90±0.67	2.85	**<0.01**
		Flower Treatment	4.22±0.65	6.53	**<0.001**
		Bee Treatment x Flower Treatment	-0.80±0.77	-1.04	0.3

Table 1 – Summary statistic for GLMM models representing the pollination (pollen remaining and pollen deposition), post-pollination (probability of pollen success and pollen tube survival) and reproductive processes (fruit set and seed set). Columns portray the model, probability distribution and link function, parameters of fixed effects and their estimates, test statistics, and significance values. Bold text in the "*P* value" column indicates statistical significance.

Model	Contrast	Ratio	SE	T statistic	*P* value
Pollen deposition	Bee Heat Plant Heat / Bee Control Plant Heat	0.47	0.18	-1.99	0.19
	Bee Heat Plant Heat / Bee Heat Plant Control	0.3	0.11	-3.16	**<0.01**
	Bee Heat Plant Heat / Bee Control Plant Control	0.13	0.05	-5.46	**<0.001**
	Bee Control Plant Heat / Bee Heat Plant Control	0.64	0.25	-1.15	0.66
	Bee Control Plant Heat / Bee Control Plant Control	0.29	0.11	-3.37	**<0.01**
	Bee Heat Plant Control / Bee Control Plant Control	0.44	0.16	-2.2	0.12
	Bee Heat / Bee Control	0.39	0.13	2.96	**<0.01**
	Plant Heat / Plant Control	0.61	0.13	4.61	**<0.001**
Probability of pollen success	Bee Heat Plant Heat / Bee Control Plant Heat	0.59	0.32	-0.97	0.77
	Bee Heat Plant Heat / Bee Heat Plant Control	0.17	0.09	-3.32	**<0.01**
	Bee Heat Plant Heat / Bee Control Plant Control	0.12	0.06	-4.06	**<0.001**
	Bee Control Plant Heat / Bee Heat Plant Control	0.3	0.15	-2.44	0.07
	Bee Control Plant Heat / Bee Control Plant Control	0.21	0.1	-3.2	**<0.01**
	Bee Heat Plant Control / Bee Control Plant Control	0.71	0.32	-0.74	0.88
	Bee Heat / Bee Control	0.65	0.23	-1.22	0.22
	Plant Heat / Plant Control	0.19	0.07	-4.56	**<0.001**
Pollen tube Survival	Bee Heat Plant Heat / Bee Control Plant Heat	0.12	0.09	-2.82	**<0.05**
	Bee Heat Plant Heat / Bee Heat Plant Control	0.02	0.02	-5.1	**<0.001**
	Bee Heat Plant Heat / Bee Control Plant Control	6.0x10-2	4.45x10-3	-7.01	**<0.001**
	Bee Control Plant Heat / Bee Heat Plant Control	0.19	0.13	-2.37	0.08
	Bee Control Plant Heat / Bee Control Plant Control	0.05	0.03	-4.3	**<0.001**
	Bee Heat Plant Control / Bee Control Plant Control	0.27	0.18	-1.9	0.21

	Bee Heat / Bee Control	0.18	0.09	-3.39	**<0.001**
	Plant Heat / Plant Control	0.03	0.02	-6.61	**<0.001**
Fruit set	Bee Heat Plant Heat / Bee Control Plant Heat	0.79	0.09	-2.04	0.17
	Bee Heat Plant Heat / Bee Heat Plant Control	0.96	0.11	-0.34	0.99
	Bee Heat Plant Heat / Bee Control Plant Control	0.72	0.08	-3.1	**<0.01**
	Bee Control Plant Heat / Bee Heat Plant Control	1.22	0.14	1.7	0.32
	Bee Control Plant Heat / Bee Control Plant Control	0.92	0.1	-0.79	0.86
	Bee Heat Plant Control / Bee Control Plant Control	0.75	0.08	-2.7	**<0.05**
	Bee Heat / Bee Control	0.94	0.07	-0.8	0.42
	Plant Heat / Plant Control	0.77	0.06	-3.34	**<0.001**
Seed set	Bee Heat Plant Heat / Bee Control Plant Heat	0.15	0.1	-2.85	**<0.05**
	Bee Heat Plant Heat / Bee Heat Plant Control	1.47x10-2	9.51x10-3	-6.53	**<0.001**
	Bee Heat Plant Heat / Bee Control Plant Control	4.92x10-3	3.10x10-3	-8.44	**<0.001**
	Bee Control Plant Heat / Bee Heat Plant Control	9.84x10-2	4.49x10-2	-5.08	**<0.001**
	Bee Control Plant Heat / Bee Control Plant Control	3.29x10-2	1.42x10-2	-7.9	**<0.001**
	Bee Heat Plant Control / Bee Control Plant Control	0.33	0.13	-2.84	**<0.05**
	Bee Heat / Bee Control	0.22	0.09	-3.89	**<0.001**
	Plant Heat / Plant Control	2.20x10-2	8.63x10-2	-9.73	**<0.001**

Table 2 – Results of post-hoc comparisons of marginal means for each level of the interaction in models. Results presented from models include pollen deposition, probability of pollen success, pollen tube survival, fruit set, and seed set. Comparisons for the pollen remaining model are omitted because there were no significant effects. Significant comparisons are indicated by bolded values in the "*P* value" column.

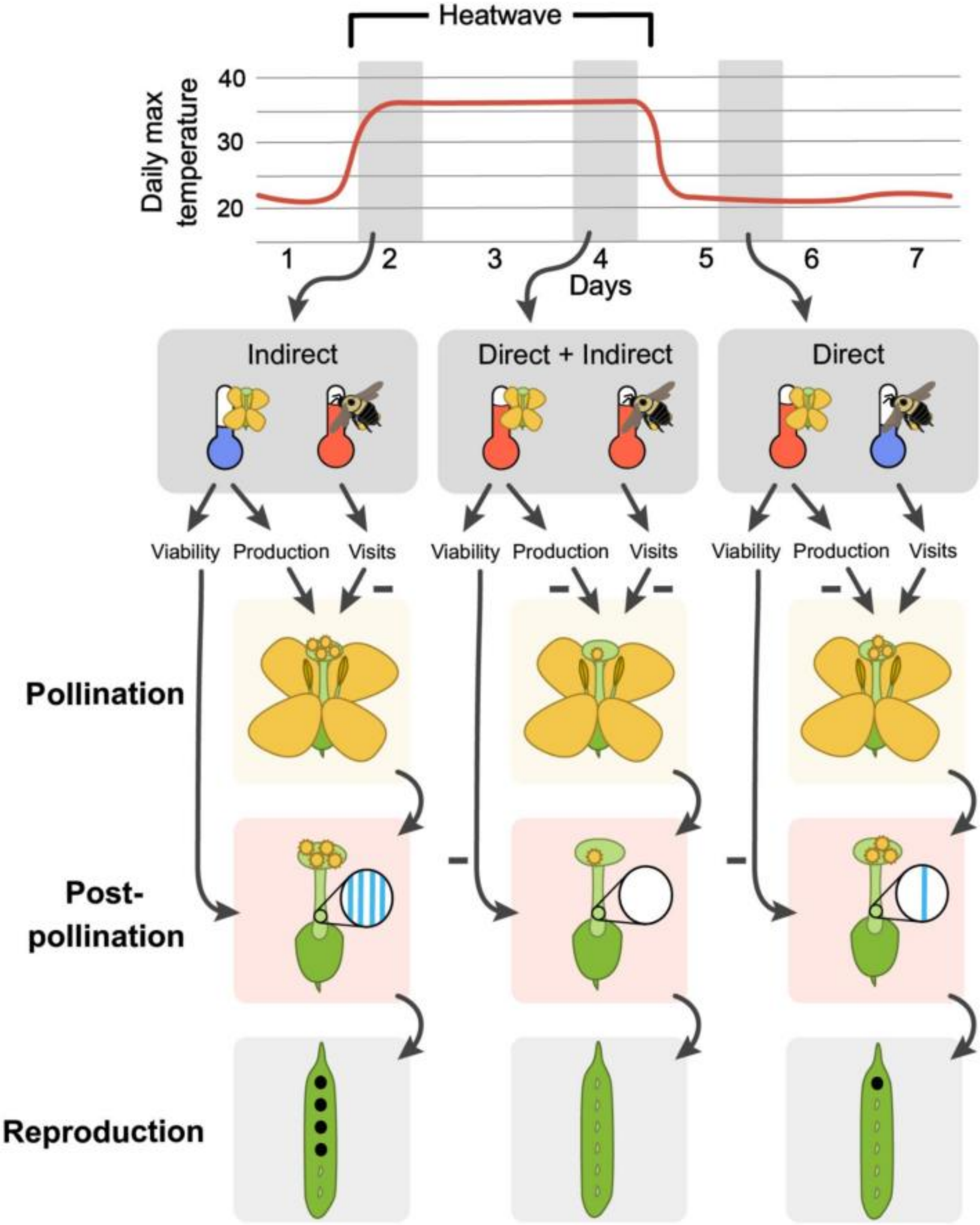
Heatwave
Daily max temperature
40
30
20
1
2
3
4
5
6
7
Days
Indirect
Direct + Indirect
Direct
Viability
Production
Visits
Viability
Production
Visits
Viability
Production
Visits
Pollination
Post-pollination
Reproduction

Figure 1: Conceptual representation of the temporal nature of a heat wave and how direct (on flower development) and indirect effects (on pollinator foraging) manifest at different periods of the heat wave and their expected outcomes for pollination (pollen deposition) and post-pollination responses (pollen viability/pollen tube survival and seed set). The left column represents pollination conditions early on in a heat wave when open flowers previously developed during normal temperature conditions, but pollinators are immediately exposed to the heat wave (Indirect effects only). The middle column represents the peak heat wave, when open flowers developed during extreme heat and pollinators simultaneously forage under extreme heat (Direct and Indirect effects). The right column represents conditions when the heat wave subsides where open flowers develop during extreme heat, but pollinators are freed from foraging under extreme heat. Pathways indicate expected responses driven by direct effects (pollen production and pollen viability), and responses driven by indirect effects (flower visits by pollinators).

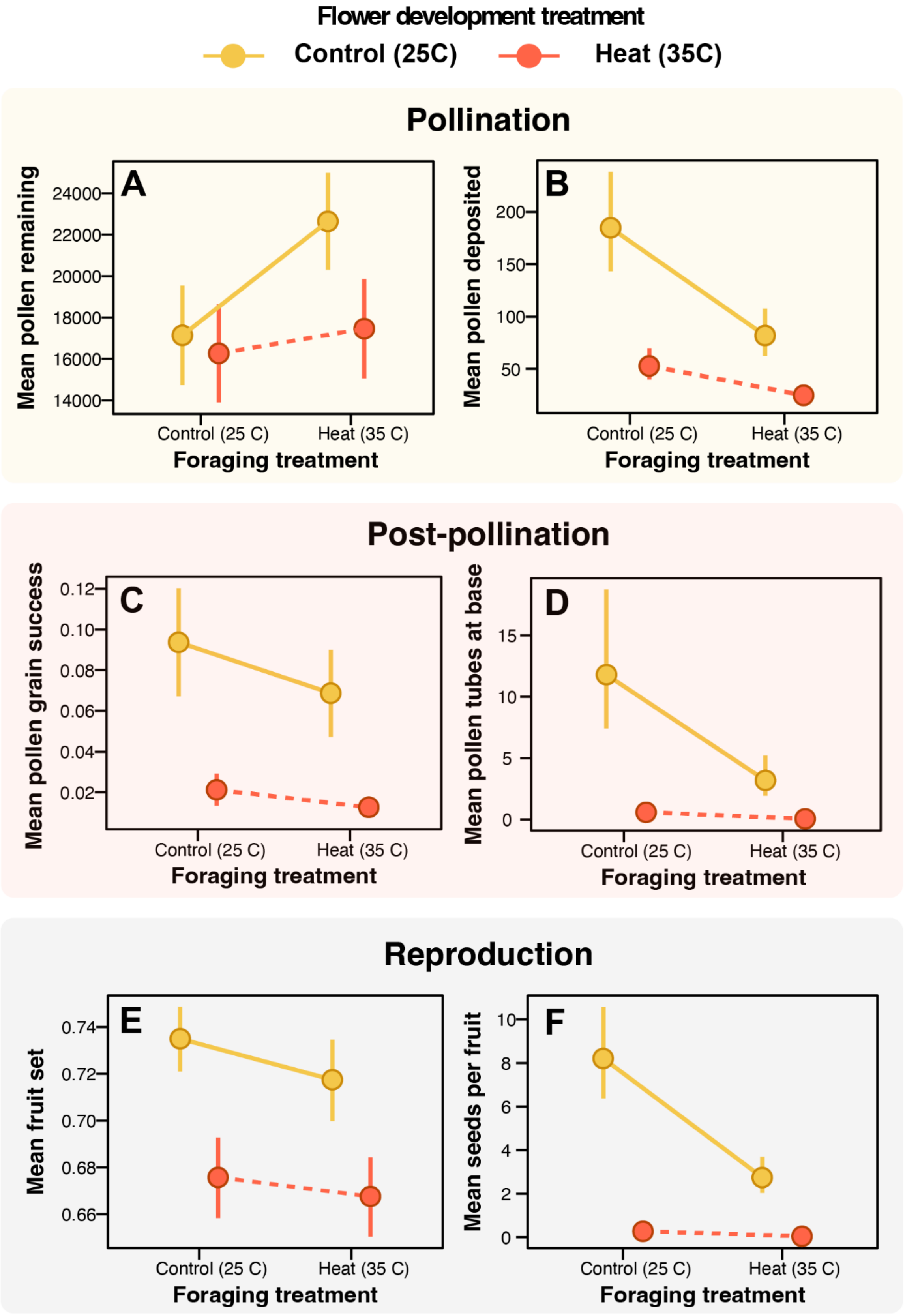
Flower development treatment
Control (25C)
Heat (35C)
Pollination
A
Mean pollen remaining
24000
22000
20000
18000
16000
14000
Control (25 C)
Heat (35 C)
Foraging treatment
B
Mean pollen deposited
200
150
100
50
Control (25 C)
Heat (35 C)
Foraging treatment
Post-pollination
C
Mean pollen grain success
0.12
0.10
0.08
0.06
0.04
0.02
Control (25 C)
Heat (35 C)
Foraging treatment
D
Mean pollen tubes at base
15
10
5
0
Control (25 C)
Heat (35 C)
Foraging treatment
Reproduction
E
Mean fruit set
0.74
0.72
0.70
0.68
0.66
Control (25 C)
Heat (35 C)
Foraging treatment
F
Mean seeds per fruit
10
8
6
4
2
0
Control (25 C)
Heat (35 C)
Foraging treatment

Figure 2: Marginal means presented as interaction plots for pollination, post-pollination, and reproductive processes in response to flower development temperature treatment and bee foraging temperature treatments. Color represents floral development temperature (Yellow: Control (25C), Red: Heat (35C), and foraging temperature treatment is on the x axis). Pollination - Panel A: depicts pollen removal as the mean number of pollen grains remaining per flower, and Panel B represents pollen deposition as the mean number of pollen grains deposited on each stigma; Post-pollination - Panel C: depicts the mean probability that an individual pollen grain produces a pollen tube that reaches the base of the style (i.e. pollen quality), Panel D: depicts the mean number of pollen tubes at the base of the style; Reproduction - Panel E: depicts the mean fruit set; Panel F: depicts the mean number of seeds produced per flower.

Supporting information

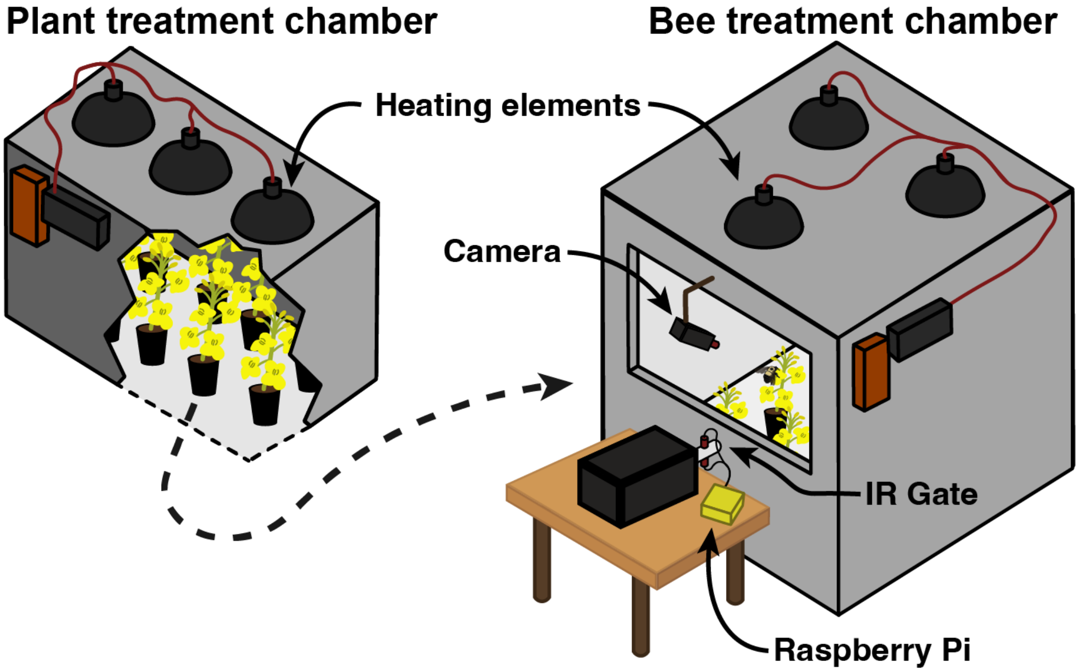

Figure S1 - Illustration of experimental chambers used for treating plants and for bumble bee foraging. In the plant treatment chamber flowers remained 72 hours before being moved into the bumble bee foraging area. The camera, infrared gate, and raspberry pi computer represented in the “Bee treatment chamber” is how pollinator visits were recorded in Hemberger et al. 2022.

Chapter 3: High temperatures cause abrupt collapse of pollination and reproduction which cannot be mediated through increased flower visitation by pollinators

Introduction

As the earth experiences intensifying climate change and instability, the frequency, intensity, and duration of heat waves is increasing (Meehl and Tebaldi 2004; Stillman 2019; Thiery *et al.* 2021). These extreme events stand to deleteriously expose organisms to temperatures beyond their thermal maxima (Ummenhofer and Meehl 2017; Ma *et al.* 2021; Harvey *et al.* 2023). When temperatures exceed organisms' thermal maxima, the high temperature often causes organism mortality (Deutsch *et al.* 2008; Vasseur *et al.* 2014). In contrast, when temperatures exceed organisms' thermal optima, they may experience fitness and performance shortfalls prior to mortality and be more vulnerable to other stressors in their environment (Deutsch *et al.* 2008; Vasseur *et al.* 2014). Given that increased heat wave frequency and intensity from climate change should expose organisms more frequently to temperature extremes, understanding how heat may affect their fitness and performance, and their interactions with other organisms is essential to inform species-level responses to climate change.

One interaction that may be especially vulnerable to the impacts of extreme heat is that between a plant and its pollinators (Hedhly *et al.* 2009; Walters *et al.* 2022; Hemberger *et al.* 2023). Plant-pollinator interactions are vulnerable to the consequences of extreme heat because of their mutualistic nature, and so fitness shortcomings may cascade from the affected individual to the mutualistic partner (Cruz *et al.* 2023). Extreme heat may decrease the quality of plant-pollinator interactions by compromising a pollinator's ability to visit flowers (Walters *et al.* 2022; Hemberger *et al.* 2023; Cruz *et al.* 2023), and the integrity of the flowers they visit including flower gametes (Hedhly *et al.* 2009; Hedhly 2011; Distefano *et al.* 2018) and floral rewards (Descamps *et al.* 2021; Hemberger *et al.* 2023). Importantly, these combined effects on visitors and plants are likely to compromise pollination directly through effects on flowers during development and indirectly through pollinator foraging (Figure 1 - Chapter 2). When extreme heat impacts flowers and their pollinators, it may lead to pollination shortfalls and decrease plant reproductive success. Recent work suggests that extreme heat may limit pollinator foraging when heat pushes foraging pollinators beyond their thermal optima (Hemberger *et al.* 2023) and leads to fewer flower visits. Through this pathway, pollinators are likely to disperse fewer pollen grains and impose a quantitative bottleneck on floral reproduction. Extreme heat can also affect flowers by reducing the quantity of male gametes produced, and their quality post-pollination (Chapter 1, Chapter 2, see also Young *et al.* 2004; Hedhly 2011). Flowers commonly respond to heat stress during floral development by producing fewer, lower-quality gametes. Fewer gametes produced can quantitatively reduce pollen that is available for deposition during pollination, whereas effects on quality can affect a pollen grain's viability or the growth rate of each pollen grain's pollen tube following pollination (post-pollination). These responses of flowers and their gametes to heat are broadly evident among plant taxa (e.g. Brassicaeae; Young *et al.* 2004, Rosaceae: Hedhly *et al.* 2005, Rutaceae: Distefano *et al.* 2018, Fabaceae; Bishop *et al.* 2016), although specific temperature thresholds for heat varies by taxa (Hedhly 2011).

Empirical studies that have evaluated the impact of extreme heat on flowers and pollinators have found large negative effects of heat, but these studies often are limited to comparisons between a few temperature treatments (Hedhly *et al.* 2009; Hedhly 2011; Bishop *et al.* 2016; Hemberger *et al.* 2023) due to the intractability of conducting experiments across the entirety of an organism's thermal performance curve. Because of these logistical constraints, understanding broader responses of pollination to the effects imposed by heat on flowers and pollinators may be more easily explored using ecological models. Previous work extensively described and modelled the thermal performance curve of organisms, creating the non-linear framework in which the fitness and performance of an organism responds to temperature (Deutsch *et al.* 2008; Vasseur *et al.* 2014). In addition, the process of pollination (pollen removal, transport and deposition) has been extensively studied and modelled (Harder and Thomson 1989; Harder 1990; Jong *et al.* 1993; Rademaker *et al.* 1997), as has as the post-pollination process (which includes pollen tube survival and ovule fertilization; Cruzan 1986, 1989; Aizen and Harder 2007; Harder *et al.* 2016). Thus, we can integrate thermal performance curves into both pollination and post-pollination to understand which stages of floral reproduction are most sensitive to heat.

I developed a model of pollination that is conceptually based on theoretical and empirical studies of the pollination process (Harder and Thompson 1989; Harder 1990; de Jong *et al.* 1993; Rademaker *et al.* 1997) and the post-pollination process (Cruzan 1986, 1989; Aizen and Harder 2007, Harder *et al.* 2016). I combined aspects of these models that may be affected by temperature through thermal performance curves (Deutsch *et al.* 2008; Vasseur *et al.* 2014) and specifically at points known to be sensitive to temperature (pollen production, pollen viability, pollen tube growth rate and pollinator visitation rate). I used this model to address a suite of questions that are challenging to conduct empirically: 1) Does increasing pollinator abundance compensate for decreased pollinator visitation per capita and pollination in response to heat?; 2) when in the pollination process and post-pollination processes is reproduction most vulnerable to heat, and do earlier-acting effects (pollen deposition) diminish the relative effect of later effects (pollen tube survival)?; 3) under what conditions are reproductive shortfalls driven by heat effects on flowers and under what conditions are they driven by heat effects acting on pollinators?

Methods

I developed a mathematical model to explore how temperature impacts plants at the pollination stage that incorporates elements of previous work on the pollination process (Harder and Thompson 1989; Harder 1990; de Jong *et al.* 1993; Rademaker *et al.* 1997). I then used an established model framework of pollen tube survival supported by numerous studies to evaluate the post-pollination stage (Cruzan 1986, 1989; Aizen and Harder 2007; Harder *et al.* 2016).

Model assumptions

My model assumes that pollination occurs over a fixed time period and calculates pollen deposition, pollen tube survival, and seed set at the end of this time period (e.g. one day). For simplicity I refer to this period as a day. Flowers can be visited by pollinators during this day, and each pollinator can make a fixed maximum number of flower visits each day. Temperature also likely affects pollination and post-pollination through both direct and indirect effects on flowers and pollinators (see Figure 1). Maximum daily temperature can affect flowers and pollinators during the pollination period by determining the number of pollen grains produced per flower, or through the number of flowers each pollinator visits on that day. My model further assumes that the effect of heat on a pollinator occurs only during pollinator foraging, and that the effect of heat occurs only during floral development. Although temperature fluctuates during a heat wave between the day and night, the effects of temperature fluctuation on floral development are not well understood. Therefore, I assume that pollinators are exposed to a constant temperature during the day. Hypothetically, pollinators could still disperse pollen in the margins of the day when temperatures are cooler, but I do not consider it here. Below I describe the thermal performance curves used in the model and each step of the model and how it integrates together.

Model structure

Thermal performance curve

Plants and pollinators respond to temperature described by a thermal performance curve outlined by Deutsch *et al.* 2008, and Vasseur *et al.* 2014 (Equation 1). In this equation the fitness (F) of an organism is a function of temperature (T) relative to its thermal optima (T_{opt}) and its thermal maxima (T_{max}), and a growth rate to the optima (σ). Fitness (F; hereafter performance) in this case is the relative performance of the organism rather than reproductive output, and so it can also apply to other processes such as pollinator foraging behavior (Fründ *et al.* 2013). Thus, in this model, the performance response is scaled between 0 and 1, with 1 at the thermal optima. Therefore, $F = 1$ is the highest performance response, and $F = 0$ is the lowest performance response. The shape of the function follows a gaussian distribution where F increases until it reaches the thermal optima at ($F = 1$). For temperatures above the thermal optima, the function changes to a parabolic function and decreases until the thermal maxima ($F = 0$):

$$F(T) = \begin{cases} e^{-((T - T_{opt})/2\sigma)^2}, & T \leq T_{opt} \\ 1 - [(T - T_{opt})/(T_{opt} - T_{max})]^2, & T > T_{opt} \end{cases} \qquad \text{Equation 1}$$

Although there are many functions for describing the thermal performance curve of organisms and their response to temperature (Schulte *et al.* 2011; Sinclair *et al.* 2016), this one is distilled down to essential components for my interests (T_{opt} and T_{max}). It captures the established biological relationship between temperature and an organisms' performance (F) which increases gradually and nonlinearly to the thermal T_{opt} and then declines sharply so that the overall performance response to temperature is asymmetric. In this particular equation, the parabolic function can produce negative values when it exceeds T_{max}. Therefore, I set the function so that $F = 0$ when T exceeds T_{max}.

Pollination model

I developed a model that is an ordinary differential equation (ODE) based on three linear models to represent pollination dynamics that can integrate the effects of the thermal performance curve to help illustrate how pollination should change on average in response to heat (developed collaboratively with S. Schreiber). I explain the relative structure of the model here but include the specific components of the ODE in Appendix 1.

The model is based on an individual flower and how it responds to visitation by pollinators. Pollination involves the removal and deposition of pollen, as a function of how much pollen is produced per flower (P), and how many visits the flower receives (v). When a pollinator visits a flower, and its body contacts the anthers it removes a proportion of the pollen from that flower's anthers (Harder and Thompson 1989; Harder 1990; de Jong *et al.* 1993, Rademaker *et al.* 1997). As the flower is successively visited, the remaining pollen is also removed proportionally (Harder 1990, Rademaker *et al.* 1997). Proportional removal means that the largest amount of pollen is removed during the first visit and decreases proportionally on subsequent visits until all pollen is removed. This pattern follows a decaying exponential function, and so the number of pollen grains remaining in the anthers of flower and the end of the time period (P_r) is a function of how many pollen grains a flower produces (P), and how many visits (v) that flower receives (Equation 2):

$$P_r = Pe^{-v} \ , \qquad \text{Equation 2}$$

In contrast, the quantity of a flower's pollen grains that pollinators collect from a flower (P_c) then saturates asymptotically, where pollen collected is a function of how much pollen the flower produced (P) and how many visits (v) it received (Equation 3).

$$P_c = P(1 - e^{-v}) \ , \qquad \text{Equation 3}$$

In the process of pollination, most of the pollen a flower produces is lost to the environment whether it falls from the pollinators or is stored in pollen-collecting structures that do not contact the stigma. Therefore, only a portion of collected pollen is deposited successfully on stigmas, and this quantity deposited is relative to how much pollen is collected (Harder 1990, Rademaker *et al.* 1997). If we assume that pollen collected is lost at a constant rate per capita of (l), and pollen is deposited at a constant per capita rate (d), then the number of pollen grains deposited by visitors per capita on the stigma of the flower (P_d) follows Equation 4. Equation 4 Equation 4 is the ordinary differential equation that also accounts for the pollen loss and deposition rate for pollen that is lost to identify total pollen grains deposited per flower.

$$P_d = \frac{dPe^{-v}(v(e^{v-d-l} - e^{v}) + d(e^{v} - 1) + l(e^{v} - 1))}{(d + l)(d + l - v)}, \qquad \text{Equation 4}$$

Post-pollination: pollen tubes

When pollen grains are deposited on the stigma of a flower (P_d) after the process of pollination, the deposited pollen grains germinate pollen tubes which must survive to the base of the style to be able to fertilize ovules. The number of pollen tubes that survive to the base of the style (P_t) depends on the number of pollen grains deposited, the growth rate of those pollen tubes (r) and the maximum number pollen tubes that can survive to the base of the style (α). These variables create a decelerating asymptotic relationship previously established by Aizen and Harder 2007.

$$P_t = \alpha\,(1 - e^{-rP_d}), \qquad \text{Equation 5}$$

Importantly, this mathematical model may produce values where the number of pollen tubes (P_t) exceeds the number of pollen grains deposited on the stigma (P_d) which is not biologically possible. Aizen and Harder solved this issue by considering the function to equal the ratio of pollen tubes to ovules (P_t/o). However, for the equation to provide more tangible information as to heat's effects, the precise pollen tube growth rate and maximum number of pollen tubes that can survive to the base of the style must be specified (Aizen and Harder 2007; Harder *et al.* 2016). Because I was instead interested in the final output to understand final pollen tube success in this sequential series of equations I constrained the model so that it could feed into a final equation of seed set to understand the final reproductive success of flowers.

Post-pollination: seed set

Ultimately the reproductive success of a flower depends on whether enough pollen tubes make it to the base of the style to fertilize available ovules. If we assume that each pollen tube that survives to the base of the style can fertilize an ovule, then the number of seeds produced (seed set) is a function of how many pollen tubes reach the base of the style and the total number of ovules (o). Any excess pollen tubes have no ovules to fertilize. If we further assume that plants are not resource limited, then the number of seeds produced (S) is a function of pollen tubes reaching the base of the style and the number of ovules (Equation 6) such that:

$$S = \begin{cases} P_t, & P_t \leq o \\ o, & P_t > o \end{cases}, \qquad \text{Equation 6}$$

Considering these equations, I connect the outputs together (Figure 2). The number of pollen grains deposited on the stigma (P_d) which is the output of Equation 4, determine the number of pollen tubes surviving to the base of the style (P_t) (Equation 5), which in turn determines how many seeds are produced (S) after the pollination and post-pollination process (Equation 6).

Modifications to the process by heat

Heat likely affects both flowers and pollinators at multiple stages during the pollination and post-pollination processes. Temperature should affect the quantity of pollen a flower produces, and its quality (as I present and discuss in Chapter 1). Gametogenesis is highly sensitive to temperature and thus temperature (Hedhly *et al.* 2009, Hedhly 2011) should change both the amount of pollen produced (quantity available to be dispersed), its viability (whether the grain can germinate a pollen tube), and the quality of germinated pollen tubes (probability a tube reaches the base of the style – what I call pollen tube growth rate).

Biologically, pollen production should influence downstream elements of the pollination process by serving as an initial constraint to plant reproductive success. Even if heat strongly impacts pollen viability and pollen tube growth downstream in the process, their relative importance depends on preceding events. For example, if temperature limits pollen grain deposition dramatically so that few pollen grains are deposited, then the effects of heat on pollen viability and pollen tube growth are only relevant to those deposited pollen grains. Thus, the relative importance by which temperature affects the post-pollination process depends on how large the effect of temperature is on the pollination process. These dependencies are captured in the model, where the pollen production parameter (P) influences the pollination model components, and the viability and quality parameters should affect post-pollination (Figure 1).

Temperature should directly affect the quantity and quality of pollen produced by a flower during floral development. Thus, I assume that pollen production and pollen quality follow the standard thermal performance relationship (Deutsch et al. 2008, Vasseur et al. 2014; Equation 1). Pollen production and pollen quality should be highest when the daily maximum temperature is close to the thermal optima ($F = 1$ when $T = T_{opt}$), and decline somewhat when below the thermal optima ($F < 1$ when $T < T_{opt}$). In contrast, because of the parabolic function of the equation above the thermal optima, pollen production and pollen quality should decline rapidly when temperatures exceed the thermal optima ($F < 1$ when $T > T_{opt}$) until it reaches the thermal maxima where pollen production should cease ($F = 0$ when $T = T_{max}$). Therefore, the number of pollen grains produced as a function of temperature is then ($PF(T)$; Figure 2). As pollen viability and pollen quality affect the post-pollination process, I assume these parameters also follow the thermal performance curve as a function of temperature, so that the number of viable deposited grains is ($P_dF(T)$), and the pollen tube growth rate is ($rF(T)$; Figure 2).

In addition to temperature's direct effects on pollen production and pollen quality, temperature should also indirectly impact pollination through the foraging of individual pollinators. Temperature can influence how many flower visits a pollinator can make within a day (v), and should follow the thermal performance curve in the same matter that pollen production and quality are affected by temperature. Thus, the total number of visits a bee can make in a day as a function of temperature is (($vF(T)$; Figure 2).

Starting constants justification

I hold some specific parameters in the model constant, basing these values on my own data from prior chapters, data from other studies, and informed guess based on my general experience with floral and bumble bee biology (Chapter 1: pollen tube maximum, and pollen tube growth rate; Chapter 2: thermal optima and maxima of flowers and bumble bees; other studies: pollen deposition and loss rate Harder 1990, Rademaker *et al.* 1997; Assumptions: ovule number, and total visits per bee). In terms of thermal performance, there is not a specific study that incrementally characterizes specific performance responses of flowers to heat across a temperature continuum. Instead, studies usually evaluate flower responses at 2-4 fixed temperatures to try and capture the range of the flower's thermal performance curve (Hedhly 2011). For example, flowers and gametes experience reduced performance in *Brassica napus* at 35C (Young *et al.* 2004; and Chapters 1 and 2). Other temperate crop plant species have been examined (Oilseed, Cereals, and Pulses), and for these species optimal temperatures ranged from 20-30C, whereas flower damaging temperatures ranged from 30-40C (Hedhly *et al*. 2009). For the sake of modelling flower responses, I assigned the flower's thermal optima (T_{opt}) at 28 C, and thermal maxima (T_{max}) at 38 C. I chose a steepness parameter for Equation 1 to be $\sigma = 30$, which flattens the curve so that temperatures below 28C negligibly reduce effects of pollen production and pollen quality. Previous work demonstrated that pollen production is not as sensitive at cooler temperatures compared to warmer temperatures (Hedhly 2011) and justifies the choice of a large σ. Bumble bee pollinators likely follow a similar thermal performance curve when it comes to foraging in field conditions (Fründ *et al.* 2013; Kenna *et al.* 2021). Although the actual critical maximum for absolute survival might be higher (Oyen and Dillon 2018), the impacts to foraging occur at much lower temperatures (Hemberger *et al.* 2023). The only study so far to experimentally evaluate temperature effects on bumble bee during foraging compared 25C and 35C (Hemberger *et al.* 2023). This study demonstrated a decline in flower visits at a constant temperature of 35C. Anecdotally, when I observed bumble bees foraging during a heat wave in Baden-Wurttemberg, Germany in August 2022, I noticed that at 35C they began to shift their behavior from foraging to a "tenting" behavior. This tenting behavior is when bumble bees hang onto the underside of leaves and cease foraging, presumably to thermoregulate. Furthermore, I also noticed that when temperatures exceeded ~38C degrees, bumble bees altogether stopped foraging. These observations also reflect bumble bee thermal performance curves in models that have been previously described (Fründ *et al.* 2013), and experimental evidence evaluating bumble bee flight at varying temperatures (Kenna *et al.* 2021). Therefore, I set the T_{opt} for foraging bumble bees to 28C and their T_{max} at 38C. I also assume that an individual bumble bee can make upwards to 300-500 flower visits per day (Neal Williams pers. comm), and so set the constant to 400 visits per day. From measuring pollen production in *B. napus* in my first chapter, I presume the maximum number of pollen grains produced per flower is ~22,000 pollen grains at T_{opt}. I also presume that 15% of the collected pollen is deposited on the stigma (d = 0.15) and that 85% of collected pollen (l=0.85) is lost to the environment based previous empirical study of pollen deposition and loss rates (Harder 1990, Rademaker *et al.* 1997).

Global Sensitivity Analysis

To test which parameters in my model had the largest effect on model output, I used sensitivity analysis. Sensitivity analysis specifically measures how much each parameter contributes to the variation in the output of a specific equation. I chose to use global sensitivity analysis instead of local sensitivity analysis because it can capture non-linear effects that are involved in my pollen deposition model, pollen tube survival model, and importantly the interactions that occur between the different parameters (Iloos et al. 2008). Specifically, I used the Sobol Jensen method of global sensitivity analysis because it is computationally the most efficient, and it is also capable of dealing with non-linear relationships between model parameters (which occur multiple times in my models; Rosolem *et al.* 2012; Renardy *et al.* 2021). To analyse global sensitivity, I used the “sensitivity” package (Iooss *et al.* 2023) in R v. 4.3.1. The “soboljensen” function uses a randomized matrix which assigns a randomized value matrix for each parameter in the equation to assess how random variation in parameters values changes the output. From this global sensitivity analysis, the simulation can determine the individual contribution of each parameter (the main effect) relative to the other parameters, and the contribution of each parameter after accounting for interactions with other parameters (the total effect).

Interaction and temperature intensity simulations:

Bee abundance and temperature:

The pollen deposition model in its current form considers a single flower, and the total number of visits that flower receives from a bee. In reality a single bee is not going to visit the same flower 400 times, so I modify the total number of visits a flower receives as a function of bee abundance and flower abundance. I then calculate average number of visits each flower receives (v_f), as a function of how many flower visits a single bee can make when responding to temperature (v_T) and the number of bees in the system (N_b), averaged across the number of flowers in the system (N_f) (Equation. 7):

$$v_f = \frac{v_T N_b}{N_f}, \qquad \text{Equation 7}$$

I retain the assumption that the distribution of visits is equal among flowers. I then simulated in the overall model how pollen deposition, proportion pollen remaining (pollen remaining / pollen produced), pollen tube survival and seed set changed as a function of temperature and bee density.

Comparing effects

Temperature can have both direct effects (via flowers) and indirect effects (via bees), on the pollination process (pollen production, and bee visitation: Equation 4) and post-pollination process (pollen grain viability, and pollen tube growth rate: Equation 5). To explore all combinations of effects and stages in the pollination process, I partitioned simulations to consist of concurrent temperature effects, flower only direct effects, and bee only indirect effects. In concurrent temperature simulations pollen production, total visits per bee, pollen grain viability, and pollen tube growth rate are all affected by the thermal performance curve. In the flower only effects, only pollen production, pollen grain viability and pollen tube growth rate were affected by temperature, which isolates simulations of direct effects. Importantly, these parameters are all being modified by the exact same thermal performance curve. In the bee only simulation, temperature effects were limited to bee visitation, which isolates simulations to indirect effects. Comparing the outputs of these simulations relativizes direct and indirect effects of temperature and their contributions to the outcomes of pollination and post-pollination processes (Figure 2).

Results

Simulation results

Each parameter modelled in the pollination and post-pollination model revealed interacting effects between bee density and temperature and the significance of direct versus indirect effects of temperature on pollination and post-pollination. Importantly, these interactive effects illuminate the non-linear nature of the processes and the context dependence of direct and indirect effects.

Concurrent Temperature

As expected, pollen deposition, pollen tube survival and seed set were low whenever bees were at low density, regardless of temperature in the concurrent effects simulation. Additionally, when bee density was low, the proportion of pollen grains remaining per flower remained high. For temperatures close to the thermal optima (28C) during the pollination process, pollen deposition increased with bee density (Figure 2A), whereas the proportion of pollen remaining decreased with bee density (Figure 2B). In contrast, as temperatures increased towards the thermal maxima (38C), pollen deposition precipitously declined beyond ~33C (Figure 2A), and pollen remaining increased beyond this threshold (Figure 2B). As expected, when temperatures exceeded the thermal maxima pollen production was 0 ($F = 0$ when $T = T_{max}$), the proportion of pollen remaining calculation produced NA values. In the post-pollination process, the number of pollen tubes surviving and seed set increased with bee density at temperatures near the thermal optima (28C; Figure 2C, D). When temperatures increased beyond the thermal optima and approached the thermal maxima, pollen tube survival and seed set precipitously declined (~33; Figure 2C, D).

Flower only effects

When only flowers were affected by heat, similar patterns emerged relative to the concurrent heat simulation. In the pollination process, pollen deposition increased with bee density when temperatures were close to the thermal optima (28C), but declined precipitously as temperatures increased beyond the thermal optima and towards the thermal maxima (~34C; Figure 2E). In contrast, the proportion of pollen remaining decreased with bee density regardless of temperature (Figure 2F). Again, as expected, when temperatures exceeded the thermal maxima, the proportion of pollen remaining produced NA values (e.g. 0/0). In the post-pollination process, pollen tube survival and seed set increased with bee density when temperatures were closer to the thermal optima, but declined rapidly when temperatures were close to the thermal maxima (~34C; Figure 2G, H).

Bee only effects

When only bee parameters were affected by temperature, I found some similar patterns to responses in Concurrent and Flower only simulations, but also some distinct differences. During the pollination process, pollen deposition increased with bee density at temperatures close the thermal optima (28C), and also as temperatures increased towards the thermal maxima before abruptly declining at the precipice of the thermal maxima (~37C; Figure 2I). The proportion of pollen remaining decreased with bee density similarly, and all pollen remained beyond the thermal maxima (38C; Figure 2J). In the post-pollination process, pollen tube survival and seed set increased with bee density at temperatures close to the thermal optima but declined abruptly at the precipice of the thermal maxima (~37C; Figure 2K, L). This abrupt change in the Bee only simulation reflects that there is still sufficient visitation for complete pollination leading right up until the bee's thermal maxima.

Global sensitivity analysis

Global sensitivity analysis revealed additional information that allowed me to interpret the outcomes of the different simulation scenarios and the differences in the relative effect of each parameter. In global sensitivity analysis the response value "relative effect size" is a measure of how much that parameter contributes to the model outcome, relative to the other parameters. Importantly, these values (between 0 and 1) do not have units, the value simply relativizes contribution of each parameter in the equation. The main effect is the contribution in the absence of interactions, and the total effect is the contribution including interactions. In the pollen deposition model, pollen production (P) had the largest relative effect size (main effect = 0.38, total effect = 0.56), whereas visits per flower and deposition rate had moderate relative effects (v main effect = 0.14, total effect = 0.22: d main effect = 0.18, total effect = 0.28, Fig 3A). Pollen loss rate (l) had a much smaller relative effect (main effect = 0.05, total effect = 0.12: Figure 3A). In the pollen tube survival model, pollen deposition (P_d) had a modest relative effect (main effect = 0.09, total effect = 0.16), whereas the maximum number of pollen tubes a style has space for (α) had the largest relative effect size (main effect = 0.73, total effect = 0.78: Figure 3B). Furthermore, the pollen tube growth rate (r) also had a marginal relative effect size (main effect = 0.11, total effect = 0.16: Figure 3B).

Discussion

Model simulations and global sensitivity analysis revealed that heat primarily limited pollination and reproduction through the production of pollen. This effect was clearest when considered with increasing bee abundance (Figure 3). Even when bee abundance increased dramatically, closer to the flower's T_{max}, pollen deposition declined when high temperatures limited pollen production. This is seemingly because there was simply not enough pollen produced by each flower to donate sufficient pollen, which lead to quantitative pollen limitation (see also Chapter 1). It is informative to consider how the proportion of pollen removed changed when bees were released from the effects of heat (flower only simulations); all pollen was removed as bee abundance increased (Figure 3F), but the number of pollen grains deposited (Equation 4: P_d) did not increase (Figure 3E). This shows that although bees made enough visits to remove all the pollen, reduced pollen production limited the quantity of pollen deposited on stigma. This interpretation is further supported by the global sensitivity analysis, for which pollen production (P) had the largest main effect in the model (Figure 4A). Because pollen production's main effect was larger than total visits per bee (v) it explains why increasing bee abundance did not lead to higher pollen deposition at high temperatures (Figure 4A).

The large effects of high temperature during the pollination process diminished the relative effect sizes of effects in the post-pollination process, which indicates the high risk of pollen quantity limitation rather than pollen quality limitation in high temperature scenarios. This diminishing effect of post-pollination processes was likely caused by the interactive nature of non-linear response in the thermal performance curve, and the non-linear nature of pollen tube survival. Because the number of pollen grains deposited (P_d) decreased in simulations at high temperatures, the values of pollen grains deposited being fed into the pollen tube survival model were lower. When pollen deposition was low in the pollen tube survival model, the effect of the pollen tube growth parameter on the output was smaller than at high pollen deposition. Biologically there is essentially less crowding of pollen tubes in the style. Essentially, when there are few pollen grains deposited, those pollen grains are largely released from pollen tube competition and the density dependence of the response (Harder *et al.* 2016). This is further confirmed by global sensitivity analysis of the pollen tube survival model, where the maximum space for pollen tubes in the style (α) had the largest effect in the global sensitivity analysis (Figure 3B), and this parameter's effect is stronger at medium to high pollen deposition (Chapter 1).

Priority effects and dominant effects – bottlenecks

In sequential biological processes or demographic processes such as floral reproduction (pollination, post-pollination, and seed production), strong effects at earlier stages can diminish the relative importance of strong downstream effects (Pepin 1991). In our case, heat's effect on pollen production during the pollination phase of the model (pollen removal and pollen deposition) had the largest effect on successful reproduction (Figure 3A, E; Figure 4A), rather than the post-pollination phase of the model. Although we expected heat to have a strong effect on pollen quality (r: pollen tube growth rate), strong impacts on pollen quality (high temperature) coincided with low pollen deposition because heat affected pollen production and quality simultaneously. We know that pollen tube survival decelerated asymptotically in response to pollen deposition, and so density dependent effects were modest at low levels of pollen deposition (Aizen and Harder 2007, Harder *et al.* 2016, Chapter 1). Thus, although heat's effects on pollen quality during development was strong (Chapter 1) its effect only manifested when pollen deposition was low, and therefore heat effects on pollen production more strongly constrained reproduction. This mathematical insight illuminates the process and results observed from the foraging chamber experiment in Chapter 2. In those experiments, heat had large effect sizes during the pollination stage (55-87% reduction in pollen grains deposited), and large effects post-pollination (80% reduction in deposited pollen grain success). If we consider the quantitative reduction (the number of pollen grains deposited) and compare it to the qualitative reduction (success of deposited pollen grains), this becomes more apparent. The mean number of pollen grains deposited in control conditions was ~200. When only plants were affected by heat, the number of pollen grains deposited decreased by 71%. Therefore, ~58 pollen grains were deposited on average per stigma in heat stressed plants which dropped 142 pollen grains relative to the control. Of those ~58 deposited pollen grains, heat reduced the success of deposited pollen grain by 80%, and so eliminated the reproductive potential of ~46 of those ~58 deposited pollen grains. While the effect of heat on deposited pollen grain success was large, the actual effect of heat on the pollination process was approximately 3 times larger (142 pollen grains vs 46 pollen grains). This is in line with our simulation model showing that heat more broadly limited reproduction through the pollination process (pollen removal and pollen deposition), than post-pollination processes.

Importance of direct and indirect effects during heat wave progression

During heat waves the importance of direct and indirect effects on the outcome of pollination may change over the temporal progression of a heat wave, and this may also depend on the intensity of the heat wave. My results revealed multiple threshold effects that can emerge during a heat wave driven by direct and indirect effects, and these effects depended on the intensity of the interaction (bee abundance) and the intensity of the heat wave (temperature). The direct effects of heat on flowers manifested during the peak of a heat wave, and post-heat wave as heat affected flowers continued to open (Hemberger *et al.* 2023; Chapter 2: Figure 1). In contrast, the indirect effects of heat on pollination through pollinators emerged at the onset of the heat wave and during the peak of the heat wave (Hemberger *et al.* 2023, Chapter 2). Here the model revealed that pollen production (a direct effect) more dramatically limited pollination and reproduction (Figure 3, and Figure 4), than visits per flower (indirect effect). Therefore, we might expect that reproduction will be most vulnerable during the peak of a heat wave and post-heat wave. This vulnerability of pollination will also depend on the intensity of the heat wave (maximum temperature), where modest heat waves may still limit reproduction through direct heat effects. In contrast, the importance of indirect effects on reproduction tends to only occur when the intensity of the heat wave is high. This suggests overall that mild to modest heat waves may still support reasonable levels of reproductive success for flowers that are receptive and pollinated during them. However, intense heat waves (temperatures close to thermal maxima), have the potential to prevent reproduction due to heat's direct and indirect effects on flowers and pollinators.

Conclusion

The abrupt, negative nature by which organism performance responds to heat is highly evident in my model of pollination, and the effects of heat at each stage of pollination and post-pollination have additive negative effects on the reproductive outcome of flowers. The results underscore how heat's direct effects on the production and quality of pollen grains lead to a predominantly quantitative shortfall in pollination for successful reproduction (see also Chapter 2). Importantly, the models also revealed that although the indirect effects of heat on pollination through pollinator visitation can constrain pollination, increasing the density of pollinators or increasing the total number of visits to flowers cannot offset constraints from heat's direct effects on flowers. Furthermore, heat's effects at earlier stages of pollination process can diminish the relative effect of heat downstream on post-pollination processes. Finally, during heat waves of high intensity direct effects on flowers will more broadly limit pollination than indirect effects. It is likely that pollination and reproduction can remain resilient when heat waves are moderate in intensity, but there is little flexibility for plants to reproduce in periods of extreme heat. Given ongoing climate change and increased frequency of extreme heat waves (Meehl and Talbaldi 2004; Stillman 2019; Thiery *et al.* 2021) our results indicate that prolonged, extreme heat coinciding with flowering will result in broad failures in floral reproduction driven by direct and indirect effects on flowers and pollinators, potentially limiting the persistence of flowering plants (Walters *et al.* 2022; Hemberger *et al.* 2023; McDonald *et al.* 2023)) and the security of food production (Battisti and Naylor 2009; Lesk *et al.* 2016).

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Model	Parameter name	Parameter	Value	Source
Pollen deposition	Max pollen production	P	22,000	Chapter 1
	Total visits per bee	v	400	Neal Williams pers. comm.
	Pollen deposition rate	d	0.15	Rademaker *et al.* 1997
	Pollen loss rate	l	0.85	Rademaker *et al.* 1997
Pollen tube survival	Pollen tube growth rate	r	0.006	Chapter 1
	Max pollen tubes	α	25	Chapter 1
Seed set	Ovule number	o	20	Chapter 1

Table 1 – Table indicating fixed parameter values used in the model and the data origin of parameter values.

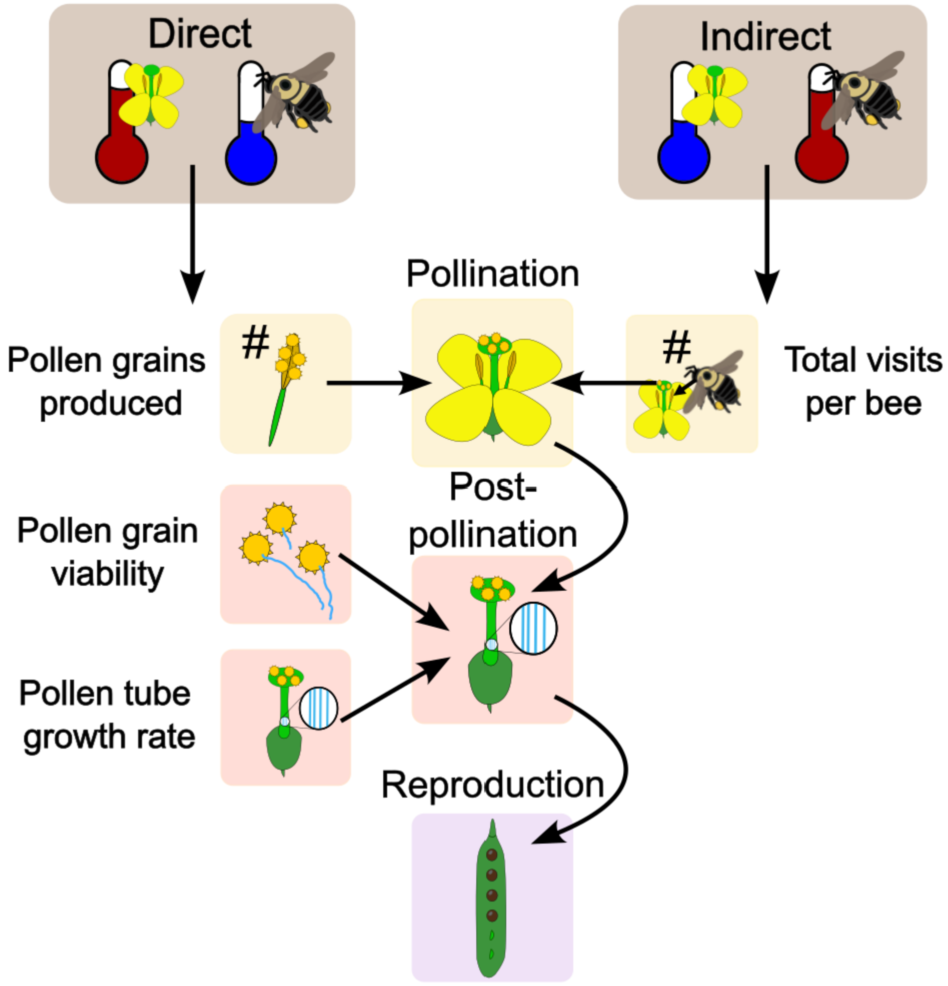

Figure 1 – Conceptual figure demonstrating the pathways by which temperatures outside the thermal optima can have direct (via flowers) and indirect (via pollinators) effects on the pollination and post-pollination processes to influence reproduction. Yellow indicates pathways by which pollination can be affected, pink indicates pathways by which post-pollination can be affected, and purple indicates the culmination of these effects on reproduction.

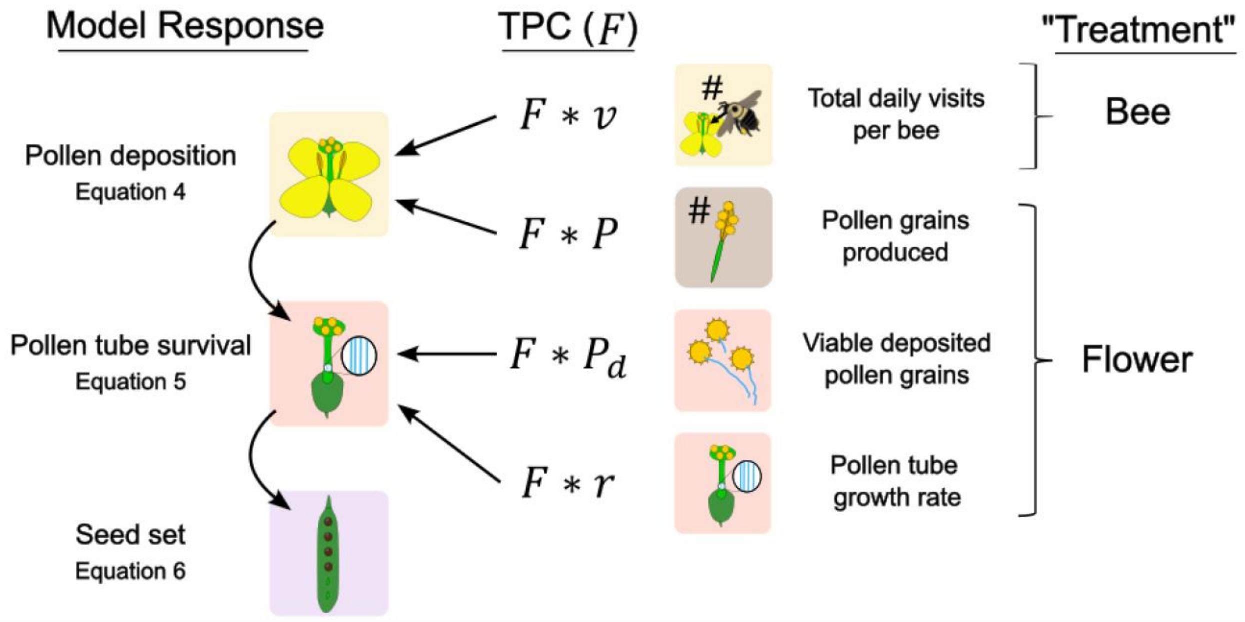

Figure 2 – Model responses and which parameters for each model response are affected by temperature along a thermal performance curve (TPC), and which of those parameters are related to bees, and which are related to plants. *F* is the value calculated per temperature using the TPC function Equation 1. For each temperature, the calculated *F* is multiplied by the specific parameters.

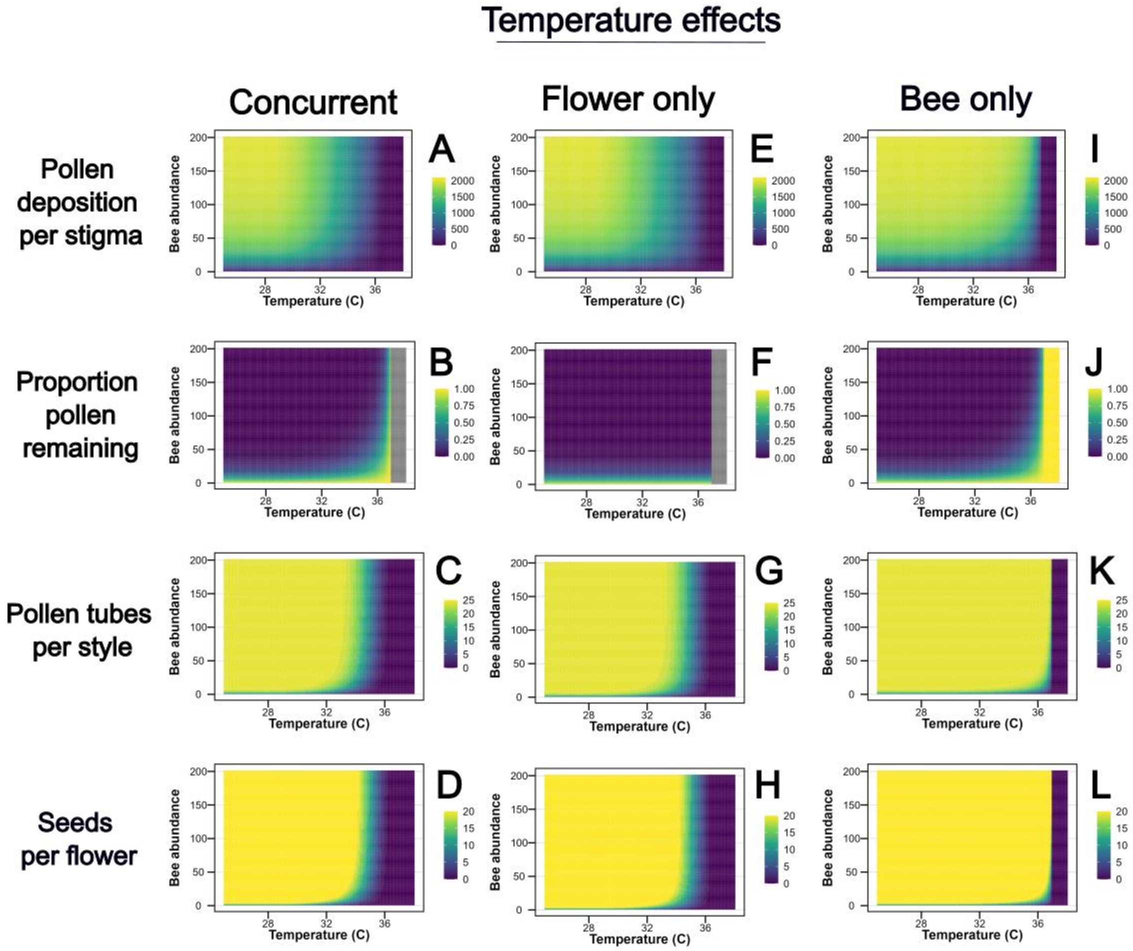

Figure 3 – Plots portray responses of model to differences in bee abundance and temperature. Columns represent how the model was run in relation to heat (Concurrent, Flower only, and Bee only). Rows represent response variables from the model (pollen deposition per stigma, proportion pollen remaining, pollen tubes per style, and seeds per flower). The color on the panel indicates the level of response as a function of bee abundance and temperature. Yellow represents high values and purple represents low values.

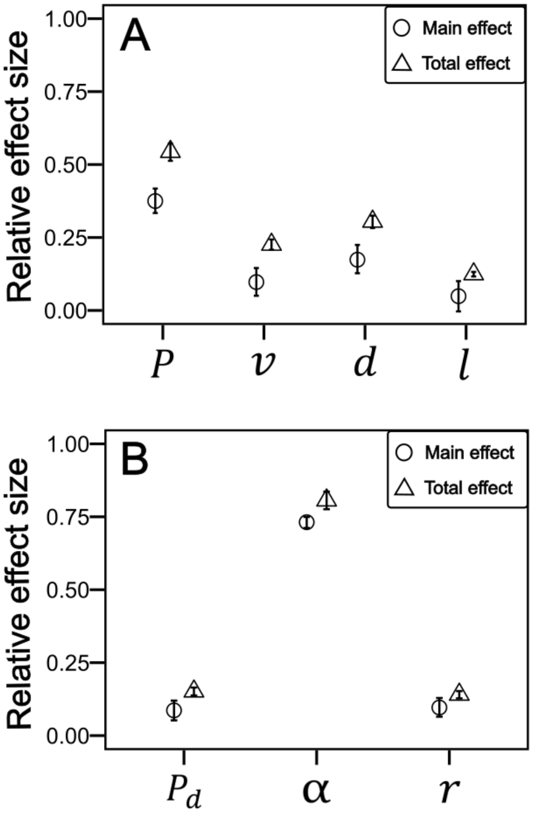

Figure 4 – Portrays the relative effect sizes of each parameter and the 95% confidence interval of each from the Sobol global sensitivity analysis. Panel A represents the effect for the pollen depositions model, where P is the number of pollen grains produced, v is the number visits a flower receives, d is the deposition rate, and l is the loss rate. Panel B represents the effects from the pollen tube survival model, where P_d is pollen deposited, α is the maximum number of pollen tubes the style can support, and r is the pollen tube growth rate.

Appendix 1

Collaboratively with Sebastian Schreiber, we developed a series of linear models to capture different aspects of the pollination process to produce Equation 4 in the main text. Specifically, the linear models capture essential aspects of the pollination process expressed as per capita rates of pollen collection, pollen deposition, and pollen loss (Figure A1). If we consider these models as continuous time models, the amount of pollen remaining in the pool at x' is equal to the amount of pollen multiplied by the effective per capita visitation rate a (Equation A1):

$$x' = -ax \text{ , Equation A1}$$

The amount of pollen collected that can be deposited in contrast considers the total pollen x, and the effective per capita visitation a determines the quantity of pollen grains collected y where some quantity of those collected those pollen grains are deposited b and some quantity of collected pollen grains are lost to the environment c (Equation A2):

$$y' = ax - (b + c)y \text{ , Equation A2}$$

We assumed that pollen lost c includes both pollen lost during flower handling and/or lost to the body of the pollinator that that collected it (i.e. pollen collected for provisioning and moved during pollen grooming). From this we can then understand that the amount of pollen that is deposited on a stigma z' is a multiplication of the per capita rate of deposition b multiplied by the number of pollen grains collected y (Equation A3):

$$z' = by \text{ , Equation A3}$$

From the development of these 3 equations, we then created the ordinary differential equation which specifically solves for the number of deposited pollen grains using the Equations A1, A2, and A3 in an Eigenvalue Matrix (Equation A4):

$$\begin{bmatrix} x' \\ y' \\ z' \end{bmatrix} = \begin{bmatrix} -a & 0 & 0 \\ a & -b-c & 0 \\ 0 & b & 0 \end{bmatrix} \begin{bmatrix} x \\ y \\ z \end{bmatrix}, \quad \text{Equation 4A}$$

We then used Wolfram Alpha to calculate the matrix exponential for this Eigenvalue matrix to produce the ordinary differential equation (ODE) used in Equation 4 in the main text. This equation originates from the ODE produced in the bottom left corner of the eigen value matrix. I took this ODE and present it as Equation 4 in the main text, but I changed the symbols used in the parameters to be more intuitive for the biology of the system. Pollen available per capita *x* is instead of *P*, *a* which equals per capita visitation is instead *v*, *b* which equals the per capita pollen deposition rate is instead *d*, and *c* which equals the per capita pollen loss rate is instead *l*.

Even though this model uses a continuous time framework, we are concerned with the outcome at the end of 1 day and are not concerned with the order in which the pollination occurs. Therefore, I can vary the parameters of interest described in the main paper (effective visitation rate and temperature for Equation 1) to understand the outcome at the end of this period.

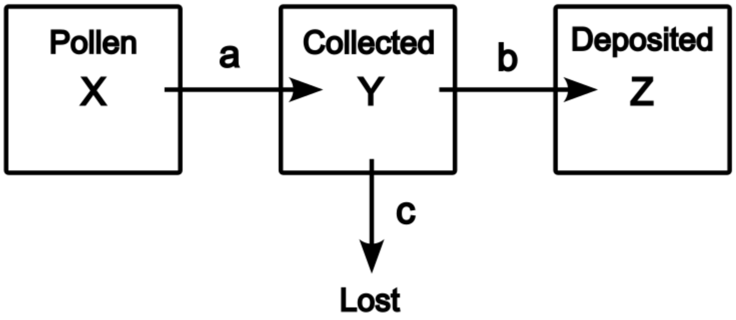

Figure A1 – Demonstrates the relative process involved in the equation that is capture by Equation 4. “X” represents the pool of pollen, of which “a” is the effective per capita visitation, which determines how much pollen is collected “Y”. From the pollen collected by pollinators some quantity of that pollen is deposited “b” on the stigma of flowers Z, and some quantity of that pollen is lost to the environment “c”.