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暖化對蜜蜂授粉行為及蜜蜂幼蟲發育的影響

Warming-induced changes in foraging behavior and the fitness consequence on larval development in honey bees

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暖化對蜜蜂授粉行為及蜜蜂幼蟲發育的影響

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本論文係張明陽君（學號R08B44016）在國立臺灣大學生態學與演化生物學研究所完成之碩士學位論文，於民國111年6月27日承下列考試委員審查通過及口試及格，特此證明

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摘要

授粉者提供重要的生態系統服務，其中全世界有三分之一以上的農作物依賴動物授粉。研究顯示，暖化導致授粉者活動和植物開花時間發生物候變化，這可能會改變當前的植物-授粉者交互作用。但是，暖化在一天的尺度中對植物-授粉者交互作用的影響尚不清楚。本研究利用西洋蜂（*Apis mellifera*）擔任授粉者，探討暖化是否影響（1）一天中蜜蜂覓食的模式、（2）一天中植物開花的時間、（3）蜜蜂採集花粉的組成，以及後續蜜蜂幼蟲的發育情況。我們透過野外實驗加熱蜂箱，以研究暖化對蜜蜂覓食行為的影響，並利用分子鑑定確認蜜蜂花粉種類後，選擇主要的植物物種來探討暖化對一天中開花時間的影響。最後，我們利用研究室飼養實驗比較暖化下的花粉組成是否會影響蜜蜂幼蟲的發育。實驗結果顯示，暖化促進並提早一天中蜜蜂的覓食活動和 *Bidens pilosa* 的開花時間，增加 *B. pilosa* 在蜜蜂花粉中的比例，並促進了幼蟲的生長。綜合以上結果，本研究顯示暖化可以通過短期的時間尺度影響授粉者的行為和植物開花時間，進而改變授粉者與植物間的交互作用以及授粉者的發育。

關鍵字：暖化、授粉者及植物間交互作用、一天內的變因、開花、蜜蜂花粉

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Abstract

Pollinators provide critical ecosystem services, such that more than one third of crops depend on animal pollination worldwide. Studies have shown that climate warming leads to phenology shifts in pollinator foraging and plant flowering at seasonal scales, which could interrupt current plant-pollinator interactions. However, warming impact on within-day patterns in plant-pollinator interactions remains unclear. Using honey bee (*Apis mellifera*) as a model pollinator, this study aims to examine if warming affects (1) the within-day pattern in honey bee foraging, (2) the within-day pattern of plant flowering onset (i.e., flowering time of the day), and (3) the composition of pollen collected by honey bees, which may influence honey bee larval development. To do so, we experimentally heated up beehives to investigate the effect of warming on honey bee foraging behavior. After identifying the bee pollen, we selected a key plant species (*Bidens pilosa* var. *radiata*) and examined warming impact on its onset of flowering. Furthermore, we investigated if any warming-induced changes in pollen composition affect larval development. Our results showed that warming advanced the within-day foraging activity of honey bees and flowering onset of *B. pilosa*, which would lead to an increase of *B. pilosa* in bee pollen composition and better bee larval development. Together, this study suggests important but overlooked mechanisms for climate change impact on plant-pollinator interactions: warming can affect the within-day patterns in pollinator behavior and plant flowering onsets, thus indirectly affecting pollinator performance.

Keywords: warming, plant–pollinator interaction, daily variation, flowering, bee pollen

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Introduction

Importance of pollinators and honey bee

Pollinators play an important role in ecosystems because they provide pollination service (Klein *et al.* 2006), biodiversity support (Ollerton 2017), and ecosystem stability (Potts *et al.* 2010). On a global scale, pollination service benefits 35% and 87.5% of crop species and flowering plants, respectively (Klein *et al.* 2006; Ollerton *et al.* 2011). Without pollinators, more than one third of the plants could not set seed and reproduce (Rodger *et al.* 2021), likely resulting in food shortage (Smith *et al.* 2015); poor plant reproduction could also lead to fewer offspring and a subsequent reduction in pollen and nectar, creating a vicious cycle between plants and pollinators (Kearns *et al.* 1998). Among pollinators, bees (Hymenoptera: Apidae) are the most important group providing ecological and economic values worldwide (Hristov *et al.* 2020). In particular, the honey bee *Apis mellifera* appears to be the most important species of pollinator in both natural and agricultural ecosystems because it has wide distribution, generalist foraging behavior and high efficiency (Hung et al. 2018). For example, *A. mellifera* contributes to the average 13.79% of floral visits in natural systems, more than double the contribution of all bumblebee species (Apidae: *Bombus*), which are widely considered as important pollinators (Hung et al. 2018). Furthermore, among the $15.12 billion economic value of crops that depend on insect pollination in the United States in 2009, honey bees and non-*Apis* pollinators contributed $11.68 billion and $3.44 billion, respectively, to the overall vale, highlighting the importance of honey bees in agriculture (Calderone 2012).

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While plant-pollinator interactions are critical to both natural and agricultural systems, global warming may interrupt current plant-pollinator interactions via creating phenological mismatches, e.g., a disruption of the overlap between pollinators and flowers in seasonal timing (Memmott *et al.* 2007; Hegland *et al.* 2009; Gérard *et al.* 2020). Pollinators and plants must match in terms of phenology in order to effectively provide pollination and produce seed set (Kudo *et al.* 2008). However, climate warming may accelerate the emergence of plant flowering and pollinator foraging at different pace, e.g., one and two months earlier for flowering and foraging in spring, respectively (Menzel *et al.* 2006; Bartomeus *et al.* 2011). This mismatch will likely reduce pollination efficiency and lead to a decline in seed set (Kudo & Ida 2013). While this warming-induced mismatch in phenology has drawn a lot of attention, studies on this topic mainly focus on warming impact on plant-pollinator interactions at seasonal scales (Kudo 2014; Kudo & Cooper 2019), and less is known about the impact at short temporal scales, e.g., within-day patterns in pollinator activity, pollen collection, and flowering onset (but see Jagadish *et al.* 2007). What also remains understudied is the consequence of such short temporal scale changes on pollinator performance (e.g., larval growth). Since consumer fitness depends on the degree of temporal synchronization between consumers and their resources (e.g., pollinators and flowers) according to the match/mismatch hypothesis (Cushing 1969, 1990; Winder & Schindler 2004), it is possible that warming impact on pollinators' within-day pattern in pollen collection may affect pollinator performance.

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While many factors affect pollinator foraging behavior (Abou-Shaara 2014), warming has become a point of interest in the Anthropocene. Observational studies have suggested that ambient temperature affects bee foraging behaviour. For example, at the relatively low ambient temperatures (e.g., 14ºC to 21ºC), the number of bees leaving the hive for foraging increased with ambient temperature (Reddy *et al.* 2015). However, at the relatively high ambient temperatures (e.g., 27ºC and 34ºC), the number of bees leaving the hive for foraging reduced with increasing ambient temperature (Reddy *et al.* 2015). Furthermore, the materials collected by foraging bees (e.g., pollen, nectar, water, and resin) may also be affected by ambient temperature. For instance, foraging bees reportedly collected more water under high temperature to regulate hive temperature (Lindauer 1955). Although the aforementioned observational studies suggest that increasing temperature (warming) will affect pollinator foraging behaviour, field experimental warming experiments would be required to verify such effect and demonstrate whether this effect varies within the day (e.g., stronger effect in the early morning due to higher bee activity). In addition, feeding experiments will be needed to examine the consequence on pollinator populations (e.g., bee larval growth).

Aims

To help understand warming impact on plant-pollinator interactions at a shorter temporal scale (e.g., within-day patterns) and the consequence on pollinators, this study focused on the honey bee *A. mellifera*, one of the most important pollinators, and used both field and laboratory experiments to answer these questions (see Figure 1): (1) Field

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warming experiment on honey bees - Within the day, will warming affect honey bee foraging behavior, such as number of foragers, amount of pollen collected by honey bees (bee pollen), individual foraging efficiency, and composition of pollen collected by honey bees? (2) Laboratory warming experiment on flowering onset – Will warming affect the timing of flowering onset within the day? (3) Larval feeding experiment – How will warming-mediated change in the composition of bee pollen influence the larval development of honey bees? Given that *A. mellifera* can respond to increasing temperatures through behavior adaptation, which varies with ambient temperature (Southwick & Heldmaier 1987; Reddy *et al.* 2015; Abou-Shaara *et al.* 2017), we expected that bee foraging behavior within a day would increase or reduce under warming when ambient temperature is below or above, respectively, the optimal range for bees. In other words, such warming effect might vary within the day because ambient temperature fluctuates hourly (e.g., interactive effect of warming and hour on foraging behavior). Since plants may flower earlier within the day under high temperature (Jagadish *et al.* 2007), we expected that warming would result in earlier flowering onset within the day. Taken together, warming-induced changes in bee foraging behavior and flowering onset within the day would likely affect the composition of bee pollen. According to the match/mismatch hypothesis, this warming-induced changes in bee pollen may consequently affect bee larval development because different pollen composition may represent different diet quality for honey bees (Donkersley *et al.* 2017; Di Pasquale *et al.* 2013).

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Materials and Methods

1. Field warming experiment on honey bees

Beehive temperature and honey bee foraging behaviors

The field warming experiment was conducted at the National Taiwan University Farm, Taipei, Taiwan, from September to November 2020. Two beehives purchased from local beekeepers were used, and were kept apart by 15 meters to prevent communication among the colonies. To maintain the colonies, I fed the bees ad libitum sugar water and pollen substitutes in the hive once a week. To simulate warming, I used a heating mat (15×28cm, 5v, 2A, Rep-Shop®) to continuously heat one of the beehives for 20 hours on alternate days. Thus, a beehive received the warming treatment, whereas the other beehive served as the control group. The two treatments (beehives) were swapped on the next day to minimize the confounding effects of environmental variables (e.g., wind, solar radiation, resource availability) across temporal scales. Meanwhile, I used iButton temperature loggers (WatchDog B102 Temp/RH Logger) to record the temperature change inside the beehive every 5 minutes throughout the experiments. The warming treatment resulted in an average of 2.4°C increase of beehive temperatures compared to the control group ($\chi^2 = 146.93$, df = 1, $p < 0.001$; Figure 2) between 6:00 and 16:00 when the bees actively showed foraging behaviors. In addition, ambient temperature was recorded by the meteorological instrument next to the Department of Atmospheric Sciences, National Taiwan University (Taipei, Taiwan). The experiments were not conducted during rainy periods.

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The field warming experiment focused on the foragers of *A. mellifera*, which collect nectar, pollen, water and resin (Abou-Shaara 2014). To quantify foraging behaviors, they were recorded with a camera (Canon IXUS 285 HS (20.2 mega pixels)), positioned from the top of the landing platform (Figure 3). I calculated the number of foragers departing from the landing platform after exiting the beehive entrance for each beehive. The observations were made by sampling a 5-minute video every one or two hours from 6:00 to 16:00, with a total of 8 time points (Figure 4). In total, there were 212 and 127 observations of foraging behaviors for the control and warming treatments, respectively. Any observed behaviors unrelated to foraging, e.g., heat dissipation and guarding, were not considered as foraging. Bees commencing orientation flights (i.e., locating beehives), if observed, were not included. To determine population size, the number of bees on each side of the beehive foundations (four in total per beehive) was recorded by opening the beehives every week. For this purpose, photos were taken and the number of workers were calculated with the assistance of the ImageJ software (https://imagej.nih.gov/ij/).

In addition to the number of foragers, I also recorded the weight of bee pollen as an indicator of bee foraging behaviors. Two pollen traps were used to collect bee pollen from foragers of each hive from 6:00 to 16:00 (a total of 7 time periods; this number is lower than foraging observation because no pollen were collected at 6:00). In total, there were 189 and 117 observations of bee pollen for the control and warming treatments, respectively. Bee pollen of each observation period were weighed to the nearest of 0.1 mg. For each observation, I also determined the individual foraging efficiency by dividing pollen weight by the number of foragers (sampled from a 5 min video; n = 184 and 109 for the control and warming treatments, respectively). Once

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weighed, the bee pollen was stored in a refrigerator at -20°C for further investigation and species identification (see below).

Composition of pollen collected by honey bees

To identify plant species from the pollen collected by bees, I sampled the previously stored pollen from one beehive and classified them based on their external morphology using naked eye observation and under a compound microscope (Zeiss Axio Scope A1). This allowed us to make preliminary species classification according to morphological and color traits of pollen grain (Figure 5). Pollen of the same species that reached more than 5% of the total pollen weight in a given sampling period of the day were classified. The top ten plant species (83.4% of the total pollen weight; Table 1) were then investigated using molecular analyses. To validate these species with molecular investigation, 100 mg pollen samples were extracted for each of the top ten plant species using the Plant Genomic DNA Purification Kit (Protech, Taiwan), following the instruction manual. Using the extracted DNA as a template, the sequences were amplified by PCR using the universal primer pairs used for *rbc*L and *trn*H-*psb*A sequence amplification. The primer pairs used for *rbc*L were F (forward): 5'-ATGTCACCACAAACAGAGACTAAAGC-3' (Kress & Erickson 2007) and R (reverse): 5'-ATGAATGTCTACGCGGTGGACT-3' (de Vere *et al.* 2012); the primer pairs used for *trn*H-*psb*A were F: 5'-GTTATGCATGAACGTAATGCTC-3' (Sang *et al.* 1997) and R: 5'- CGCGCATGGTGGATTCACAATCC-3' (Ford *et al.* 2009). The PCR reaction settings for *rbc*L and *trn*H-*psb*A were identical, but were processed and analyzed separately. The total volume of each PCR reaction was 50 µl, including 1 µl of F primer, 1 µl of R primer, 10 µl of Fast-RunTM Taq 5x Master Mix (Protech, Taiwan), 4 µl of pollen sample DNA and 34 µl of sterilized water. Reaction conditions were (1) 9

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minutes at 94℃, (2) 40 cycles of 30 seconds at 90℃, 30 seconds at 58℃ and 40 seconds at 72℃, and (3) 7 minutes at 72℃. After the reaction, the PCR product was electrophoresed on 1.5% agarose gel, and the presence of the product and the molecular weight of the product were observed under ultraviolet light, and then the product sample was sent for Sanger sequencing analysis (Genomics, Taiwan). The bidirectional sequences were then manually aligned and debugged with Clustal Omega (Sievers *et al.* 2011) software. The primer fragments were removed, yielding the corrected *rbc*L and *trn*H-*psb*A sample sequences, and the BLASTn (Altschul *et al.* 1990) software was used to compare the similarity with the GenBank database sequences to identify plant species.

2. Laboratory warming experiment on flowering onset

In this laboratory warming experiment, *Bidens pilosa* var. *radiata* (hereafter "*Bidens pilosa*") was selected as the object to study the time of plant anthesis onset because it had the highest abundance of all bee pollen (comprising 28.0% of the total bee pollen weight; Table 1), and could be harvested throughout the field operation experiment period (Figure 6A). The seeds of *B. pilosa* were haphazardly collected from the campus of National Taiwan University and planted in a growth chamber with a constant temperature of 28℃ on a 12:12 photoperiod. On the 17th day, when most individuals were found to have grown the second trifoliate leaf, they were moved to a temperature-controlled greenhouses of phytotron with natural light. I haphazardly assigned individuals to two temperature-controlled greenhouses for continued cultivation according to the ambient daytime temperature (26.7℃; 6:00-19:00) and nighttime temperature (24.0℃; 19:00-6:00) in the field warming experiment. The day and night

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temperature of the control group was 25/20℃, whereas the day and night temperature of the warming group was 30/25℃. During the cultivation period, the individual positions were adjusted appropriately so that each individual was homogenously exposed to natural sunlight. The soil was kept moist, with growth fertilizer supplied once a week. Huabao No. 2 and No. 3 (HYPONeX®) was used to promote plant growth and bud maturation, respectively. After another 38 days, the observation started when individuals in both the control and the warming treatments had the head with mature disk flowers. Individuals that just started the first bloom or finished the last bloom were not used to ensure the time of plant anthesis onset was fully captured. The experiment included a total of 39 individuals in the control treatment and 30 individuals in the warming treatment. From 6:30 to 12:00, the number of disk flowers with emerging dehiscent anther (hereafter "number of flowering onset"; see figure 2d in Budumajji *et al.* (2018)) was recorded every half an hour. After each observation, the disk flowers alongside their stamens were removed with a pair of tweezers to facilitate the next observation (Figure 7).

3. Larval feeding experiment

To investigate warming-mediated effects on pollen composition and its ecological consequences on bee larval development, I experimentally manipulated the composition of bee pollen and fed them to bee larvae. The pollen were collected between 11-19 November 2020 during which the field warming experiment were conducted. Combined with the semi-artificial diet modified from Hendriksma *et al.* (2011), four different pollen composition treatments were formulated and fed to the larvae. These treatments were: (1) the control group (*B. pilosa* : others = 38% : 62%), (2) the warming group (*B.*

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pilosa : others = 47% : 53%), (3) the warming plus group (*B. pilosa* : others = 55% : 45%) and (4) the *B. pilosa* only group (*B. pilosa* : others = 100% : 0%) (see Table 2 and Figure 8 for complete percentage). The warming plus group generated a greater proportion of *B. pilosa* pollen, representing a more serious warming scenario, whereas the *B pilosa* only group served as the experimental control. These pollen compositions were determined according to the formula:

$$P_c = \frac{\sum_{t=1}^{i} W_t B_t}{\sum_{t=1}^{i} W_t} \quad , \quad P_w = \frac{\sum_{t=1}^{i} W_t B_{t+1}}{\sum_{t=1}^{i} W_t}$$

P_c was the weight percentage of *B. pilosa* pollen in the control group, whereas P_w was the weight percentage of *B. pilosa* pollen in the warming group. t was a certain time of day from 6:00 to 16:00 (a total of 7 time periods). W_t was the average weight of bee pollen given a certain t. W_t for both P_c and P_w were identical for all time periods except for that of 6:00, according to our pollen weight results (Figure 9). B_t was the weight percentage of *B. pilosa* in a certain time of day. The weight percentage of *B. pilosa* pollen in the warming plus group equalled to $P_w + (P_w - P_c)$. Since the percentage of other plants varied between groups, I adjusted the pollen weights of each of these species according to their ratio from the control treatment. In total, there were 18, 20, 24 and 20 individuals for the control, warming, warming plus, and *B. pilosa*-only treatments, respectively.

A side of hive foundation with sufficient eggs was chosen. I fixed the transparent plastic sheet on top of the hive frame and used a marker to record the position of each egg on the plastic sheet. To ensure similar developmental stage of bee larvae, I selected newly-hatched larvae the next day. Two days later, the hive frame containing 3rd instar larvae (n = 101) was brought back to the laboratory for further larval feeding experiment.

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According to previously established protocol, all larvae were maintained in climate-controlled environments and fed with the semi-artificial diet modified from Hendriksma *et al.* (2011).

A saturated solution of potassium sulfate (K_2SO_4) was made and added to the storage box to keep the humidity stable, with an iButton temperature logger placed inside to monitor the temperature and humidity. The temperature was maintained at 35±0.25℃ and the humidity was maintained at 95±3%. The semi-artificial diet included two different food formula weight ratios fed to the larvae as they grew. On the first day when the 3rd instar larvae were moved to the growth chamber, the larvae were fed with the diet containing 3% bee pollen, 47% royal jelly and 50% aqueous solution (3% yeast extract, 15% glucose, 15% fructose and 0.2% Nystatin (50 mg/ml; SIGMA; Biobasic)). From the second day onwards, the larvae were fed with the diet containing 3% bee pollen, 47% royal jelly and 50% aqueous solution, with the aqueous solution formula adjusted to: 4% yeast extract, 18% glucose, 18% fructose and 0.2% Nystatin (50 mg/ml). Among them, Nystatin could prevent the pollen in the formula from being infected by fungi (Veloso & Lourenço 2014). The prepared diets were divided into small tubes and stored in a -20℃ refrigerator, and were thawed and warmed to 35℃ before feeding to the larvae.

According to Hendriksma *et al.* (2011), the semi-artificial diet was fed to each larva for four consecutive days, with the volume of 20, 30, 40, 50 μL, respectively. Given that the feeding status varied between individuals, I split the diet volume into 4-6 separate times per day, adjusted the diet amount fed to the larvae and recorded the volume immediately (Figure 10). When the 5th larvae curled vertically to the plane of the hive

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foundation (prior to the prepupal stage), the larvae from the brood comb were gently collected using a pair of tweezers and weighed (to the nearest 0.1 mg). The bee growth efficiency index was determined by dividing their body mass (mg) by the actual diet volume consumed (μL).

Statistical analyses

All statistical analyses were conducted using the statistical software R version 4.1.1 (R Development Core Team 2014). Generalized linear mixed models (GLMMs) were conducted in the package *lme4* (Bates *et al.* 2015) to analyze the effects of warming on bee behavior, plant flowering, and larval development. For all comparisons, p values < 0.05 were considered statistically significant. No multicollinearity was detected in either model under model selection, with all VIFs (Variable Inflation Factors) < 5. All graphs were prepared in the package *ggplot2* (Wickham 2016).

Beehive temperature

To determine if our experimental warming was effective in elevating temperatures of bee hives in the field warming experiment, we analyzed in a GLMM bee hive temperature as a dependent variable, whereas temperature treatments (0: control, 1: warming) as a fixed effect. We also included date nested within bee hives as a random effect.

Honey bee foraging behaviors

To investigate whether warming affected bee performance, we analyzed in GLMMs number of foragers with Poisson error distribution, whereas bee pollen weight and

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individual foraging efficiency with Gaussian error distribution. Since pollen were collected hourly 6:00-10:00 and once every two hours at 10:00-12:00, 12:00-14:00, and 14:00-16:00, we standardized pollen weights per hour for the analyses. Pollen weight and individual foraging efficiency were log-transformed prior to analyses to meet the assumption of normal distribution. In all models, we included the fixed effects temperature treatments (0: control, 1: warming) as a categorical variable, the time of day as a continuous variable, and their interaction, while date nested within bee hives was included as a random effect. To account for potential non-linear relationships predicted by the time of day, the third degree was included initially but was reduced to lower degrees when appropriate. For analyses of number of foragers and bee pollen weight, we also statistically controlled for ambient temperature and colony size (scaled and centered) as continuous fixed effects. Once an interaction between warming and the time of day was detected, we analyzed the data collected at different timings separately to uncover specifically when temperature treatment had an effect.

Composition of pollen collected by honey bees

To compare differences in the bee pollen composition simulated under control and warming scenarios, we used non-metric multidimensional scaling (NMDS) using the function metaMDS, with temperature treatment and the time of day as fixed effects by using the *vegan* package (Oksanen *et al.* 2020). NMDS was based on Bray–Curtis dissimilarity to calculate the distance matrix for an ordination with 999 iterations. When applying NMDS, we constrained permutations within groups of days based on similar bee pollen composition (see Figure 6A).

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Laboratory warming experiment on flowering onset

To investigate whether warming affects plant flowering onset, we analyzed the number of flowers using a Poisson GLMM by including temperature treatments (0: control, 1: warming), the time of day, and their interaction as fixed effects. Similarly, we included the second degree of the time of day to account for potential non-linear relationships. Different *B. pilosa* individual identity was included as a random effect since each individual was sampled every 30 minutes for a total of 12 observations.

Larval feeding experiment

To test for the effect of warming-mediated pollen composition on the larval development, we analyzed the growth efficiency index (larvae weight divided by fed diet; log-transformed) in a GLMM with Gaussian distribution. Different pollen composition (i.e., control situation, warming situation, warming plus situation and *Bidens* only situation) was included as a categorical variable. We then conducted Tukey post-hoc comparisons using the *lsmeans* package (Lenth 2016) to test for differences in larval development between different pollen composition.

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Results

1. Field warming experiment on honey bees

Beehive temperature

Compared to the control group, the warming treatment increased the temperature in beehives by average 2.4°C during 06:00-16:00 (when honey bees were most active) (χ^2 = 146.93, df = 1, $p < 0.001$; Figure 2). Comparing the difference in temperature changes inside and outside the beehives, the warming of 2.4°C in the beehives is equivalent to an increase of 3.5°C in ambient temperatures (Figure 11).

Number of foragers

Our warming treatment affected the number of honey bee foragers over the time of the day ($Time^3 \times$ Warming: χ^2 = 3.98, df = 1, p = 0.046; Figure 4; Table 3). Specifically, when the data collected from different hours were analyzed separately, the warming treatment in the morning increased the number of foragers leaving out at 6:00 (χ^2 = 10.12, df = 1, p = 0.001). Furthermore, the number of foragers leaving out was high in early morning and decreased over the time of the day (χ^2 = 40.30, df = 1, $p < 0.001$).

Amount of pollen collected by honey bees

Similarly, the amount of pollen collected by honey bees (bee pollen) changed over time (Time × Warming: χ^2 = 4.63, df = 1, p = 0.031; Figure 9; Table 3). The total weight of bee pollen was highest in the early morning and decreased over time (χ^2 = 33.31, df = 1, $p < 0.001$; Figure 9). When the data collected from different hours were analyzed

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separately, the results showed that the warming treatment at 6:00 in the morning increased the weight of bee pollen (χ^2 = 5.06, df = 1, p = 0.024).

Individual foraging efficiency

Combining the results of the number of foragers and the amount of pollen collected, the results showed that the individual foraging efficiency was highest in the early morning and decreased over time (χ^2 = 11.16, df = 1, p < 0.001; Figure 12; Table 3). The individual foraging efficiency was not affected by warming (χ^2 = 0.27, df = 1, p = 0.603).

Composition of pollen collected by honey bees

NMDS analysis showed that the composition of bee pollen varied with time (F = 5.71, p = 0.001) but not temperature treatment (F = 1.12, p = 0.342; Figure 13). The collected pollen that accounted for at least 5% of the total pollen weight (see Materials & Methods) were identified into 75 plant species (Figure 6; Table 1). Roughly, the pollen of about 20 different plant species would be collected by bees on a daily basis (Figure 6A).

Regarding the pollen composition in terms of weight, we identified the top ten plant species with the highest weight in Table 1. Specifically, the highest was *Bidens pilosa* accounting for 28.0% of the total pollen weight, the second highest was *Koelreuteria elegans* accounting for 12.3% of the total pollen weight, and the third highest was *Bauhinia* x *blakeana* accounting for 9.8% of total pollen weight.

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Regarding the pollen composition over time, we analysed the bee pollen collected on a daily basis and found that most plant species were collected by bees during a limited time period only, likely due to their flowering period (Figure 6A). However, *B. pilosa*, which flowers year around, was collected every day during our study period (Figure 6A). We also analysed within-day variation in pollen composition (Figure 6B). Note that *B. pilosa* became the dominant species after 8:00 and remained at a high proportion before sunset (Figure 6B).

2. Laboratory warming experiment on flowering onset

The results of the laboratory warming experiment showed that the onset of flowers with dehiscent anther peaked at ca. 9:00 under the control treatment (25/20°C), while it peaked at ca. 8:00 under the warming treatment (30/25°C). This suggested that warming caused the *B. pilosa* to bloom ca. one hour earlier ($\text{Time}^2 \times$ Temperature treatment: χ^2 = 14.44, df = 1, $p < 0.001$; Table 3, Figure 14).

3. Larval feeding experiment

The larval feeding experiment showed that increased pollen proportion of *B. pilosa* altered larval development (χ^2 = 19.46, df = 3, $p < 0.001$; Table 3). Tukey post-hoc comparisons revealed no difference in the larval growth efficiencies among the warming, warming plus and *B. pilosa*-only treatments, but the larval growth efficiencies in these groups were all higher than the growth efficiency in the control group (Figure 15; Table 4).

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Discussion

While many studies have examined warming impact on plant-pollinator interactions at seasonal scales (e.g., phenology shift), it remains unclear how warming may affect the within-day patterns in plant-pollinator interactions and whether this will have a consequence in pollinator performance. To answer these questions, this study conducted both field and laboratory experiments and showed the following results: (1) Warming on bee hives by 2.4°C affected bee foraging behavior within the day, by increasing the number of foragers going out (Figure 4) and the weight of pollen brought back by foragers in the early morning (Figure 9). The increase in bee pollen should be mainly due to the increased number of foragers, since warming did not affect the individual foraging efficiency of bees (Figure 12). Furthermore, warming did not affect the composition of bee pollen, with *B. pilosa* as the most important pollen source (28.0% of the total pollen weight) (Figure 13; Table 1). The composition of bee pollen, however, changed with time of the day (Figure 13). (2) Warming by 5°C advanced the onset of *B. pilosa* flowers, leading to pollen release about an hour earlier (Figure 14). This would suggest that bees may be able to collect their most important pollen source (i.e., *B. pilosa*) an hour earlier, likely resulting in a higher proportion of *B. pilosa* in bee pollen collected in the morning. (3) Taken together, we examined if such warming-mediated change in bee pollen composition (i.e., an increase of *B. pilosa*) affects bee larval growth. The results showed that increasing *B. pilosa* pollen in percentage under warming scenario increased the growth efficiency of bee larvae (Figure 15). We further discuss warming impact on within-day patterns in pollinator foraging, warming impact on flowering onset and bee pollen composition, as well as pollen composition and bee larval growth in the following sections.

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While observational studies suggested that increasing ambient temperature would affect pollinator foraging (Reddy *et al.* 2015; Clarke & Robert 2018), our experimental warming study reveals a more complete picture: warming and time of the day can interactively affect bee foraging behaviour. Specifically, warming in this study increased the number of foragers and pollen weight only in the early morning (Figure 4; Figure 9; Table 3). The reason for the early morning effect might be due to the ambient temperature change within the day. The ambient temperature was mainly below 21°C at 6:00 in the early morning (Figure 2; Figure 11). Since bee foraging activity increases with temperature at the relatively low ambient temperature (14ºC to 21ºC) (Reddy *et al.* 2015), warming should increase bee foraging at 6:00 (Figure 4; Figure 9). Warming in this study did not inhibit bee foraging likely because the average ambient temperature was mainly below the threshold of 27ºC and 34ºC (Reddy et al. 2015). However, another study which was also conducted at National Taiwan University showed that the number of bees departing the beehive and the bee pollen count peaked at 10:00 rather than early morning as shown in this study (Ngo *et al.* 2021). These different patterns of foraging behavior can be attributed to differences in bee colonies, such that each colony is characterized by distinct personality of foraging. Future studies should consider differences in such "collective personality" in explaining warming-mediated foraging behaviors among bee colonies. Furthermore, Ngo *et al.* (2021) conducted the study from August to December, during which the seasonal change can have an important effect on bee behaviors changes, making their conclusions less comparable to ours focused on a relatively short temporal scale. Taken together, this study highlights that warming

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impact on pollinator foraging activity may depend on time of the day — an overlooked issue for assessing climate change impact on plant-pollinator interactions.

Warming impact on flowering onset and bee pollen composition

While previous studies focus on warming-induced mismatch in plant and pollinator phenology at seasonal scales (e.g., warming-induced shifts in flowering dates), this study shows a within-day mechanism for warming impact on plant-pollinator interaction: warming may advance the time of the day for flowering onset in the main pollen plants and then affect the composition of bee pollen. Because anther dehiscence is sensitive to increasing temperature (Matsui *et al.* 2001), we expect that warming-induced advancement of flowering within the day (e.g., 1 hour earlier in this study) to be common in nature. We also expect that this advancement of flowering will significantly affect the composition of pollen collected by pollinators if this shift occurs in pollinator's preferred plants. For example, in this study, *B. pilosa* was the most preferred, important pollen source (i.e., accounting for 28.0% of bee pollen), although some other plants flowered at the same time. Therefore, if *B. pilosa* flowered one hour early under warming, bees, which were more active in the morning (Figure 4; Figure 9), should be able to access this preferred pollen resource one hour earlier and therefore change the composition of bee pollen. This should occur even if other less preferred plants flower earlier as well.

The warming-induced shifts in flowering within the day and in bee pollen may have important consequences in plant-plant competition and pollinator performance. These patterns and consequences at shorter time scales have been overlooked in previous

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studies but deserve further investigation. Regarding plant-plant competition, many plants rely on bees, the generalist pollinators (Geslin *et al.* 2017), to pollinate. If warming-induced shifts in flowering time of the day provide more benefit to certain plant species (e.g., more bee visits), this may tip the original plant-plant competition status and consequently affect plant community composition. Regarding pollinator performance, more details will be discussion in the next section.

Pollen composition and bee larval growth

One of the most important, novel findings of this study is that warming-induced shifts in pollen composition collected by pollinators (bees) within the day can subsequently affect pollinator performance (bee larval growth). Previous studies have examined the effects of bee pollen diversity or nutrition on bee growth (Di Pasquale *et al.* 2013; Donkersley *et al.* 2017), but it remains unclear whether warming will affect bee pollen composition and consequently bee larval growth. The results of this study showed that an increase of *B. pilosa* in bee pollen composition under warming scenario would increase the growth efficiency of bee larvae. The results suggest an important but overlooked mechanism for warming impact: warming could influence flowering onset and bee pollen composition, thus indirectly affecting pollinator population performance.

This study revealed a better bee larval performance under a higher percentage of *B. pilosa* pollen (i.e., warming, warming plus, and *Bidens*-only treatments) (Figure 15). This is different from some of previous studies on pollen nutrients. Pollen contains nutrients such as protein, lipids and vitamins (Roulston & Cane 2000), among which protein level critically affects the lifespan of bees (Amdam & Omholt 2002), brood

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rearing (Crailsheim 1990), etc. For example, 20-23% protein content of pollen substitutes was very suitable for the dietary needs of bees (Herbert 1992). However, *B. pilosa* pollen consisted of only 16% of protein (Hsu *et al.* 2021) and could lead to lighter larval biomass (Tasei & Aupinel 2008), different from the results of this study. In addition, low diversity of bee pollen may result in poor bee larval development or health (Di Pasquale *et al.* 2013; Filipiak *et al.* 2017), different from the results of this study (i.e., *Bidens*-only treatment). Although examining the underlying mechanism is not within the scope of this study, we suspect that endosymbiotic bacteria may play a key role. Endosymbiotic bacteria of bee larvae can be obtained from nurse bees (Kwong & Moran 2016) and help larvae break down pollen wall and absorb pollen nutrients (Kešnerová *et al.* 2017). Since *B. pilosa* pollen is available year round in our study system, it might be advantageous for bees to keep related endosymbiotic bacteria to digest this predictable and preferred pollen source. This may explain the difference between this and other studies.

Potential caveats

This study has at least two potential caveats. First, this study increased bee hive temperature by heating only the top of bee hives. Therefore, our results may underestimate warming impact on bee activity. This approach is a compromise due to bee hive structure, bee behaviour, and a challenge in field heating device. A more advanced approach in the future (e.g., heating the whole bee hives and surrounding environment) might reveal the more precise results. Second, this study only examined the warming impact on flowering onset in *B. pilosa* due to logistics. Including other plant species will better reveal the potential impact of warming on bee pollen

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composition. We, however, suspect that including other plants in our laboratory study may not change the main findings since *B. pilosa* is the most preferred, important pollen source. Therefore, its shift in flowering time of the day should outweigh the shifts of other plants, if it happens.

Conclusions

While studies have examined warming impact on the phenology of pollinator foraging activity and plant flowering at seasonal scales, few studies investigate the warming impact on the within-day patterns in plant-pollinator interactions. Based on field and laboratory experiments, our results show that warming could advance pollinator foraging activity and flowering onset within the day, consequently affecting pollinator larval performance. Therefore, our study provides insights for important, but overlooked, mechanisms for climate change impact on plant-pollinator interactions.

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Table 1. All plant species and their relative percentage in bee pollen during field warming experiment.

Species ID	Percentage	Identified species
10	27.968%	*Bidens pilosa*
8	12.293%	*Koelreuteria elegans*
58	9.752%	*Bauhinia* x *blakeana*
29	8.500%	*Ulmus parvifolia*
62	5.996%	*Acacia confusa*
60	5.064%	*Mikania micrantha*
51	4.042%	*Litsea cubeba/hypophaea*
30	3.813%	*Melaleuca leucadendra/quinquenervia*
50	3.727%	*Oryza rufipogon/sativa*
63	2.191%	*Polyspora axillaris*
54	1.848%	-
61	1.456%	-
68	1.188%	-
36	1.172%	-
7	0.831%	-
52	0.820%	-
49	0.784%	-
21	0.649%	-
71	0.619%	-
9	0.462%	-
59	0.348%	-

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Table 1. (continued)

Species ID	Percentage	Identified species
56	0.312%	-
2	0.311%	-
3	0.259%	-
64	0.249%	-
26	0.239%	-
18	0.236%	-
19	0.207%	-
37	0.202%	-
33	0.164%	-
16	0.158%	-
17	0.151%	-
42	0.140%	-
55	0.139%	-
69	0.137%	-
14	0.125%	-
11	0.123%	-
13	0.117%	-
5	0.094%	-
31	0.093%	-
15	0.082%	-
70	0.079%	-
22	0.068%	-
35	0.066%	-

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Table 1. (continued)

Species ID	Percentage	Identified species
32	0.064%	-
74	0.055%	-
46	0.054%	-
66	0.051%	-
43	0.045%	-
57	0.040%	-
65	0.036%	-
53	0.035%	-
72	0.033%	-
27	0.032%	-
24	0.031%	-
73	0.029%	-
1	0.027%	-
38	0.026%	-
48	0.025%	-
23	0.024%	-
39	0.022%	-
12	0.018%	-
20	0.017%	-
47	0.016%	-
40	0.015%	-
75	0.013%	-
6	0.010%	-

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Table 1. (continued)

Species ID	Percentage	Identified species
44	0.009%	-
25	0.008%	-
67	0.003%	-
41	0.003%	-
45	0.002%	-
28	0.002%	-
4	0.002%	-
34	0.001%	-
Others	1.978%	-

Others refers to unidentified pollen by morphological traits.

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Table 2. The experimental composition of bee pollen and their percentage of the four treatments in the larval feeding experiment.

Species ID	**Control**	**Warming**	**Warming plus**	***Bidens* only**
10	38.00%	47.00%	55.00%	100.00%
60	15.33%	13.10%	11.12%	0.00%
62	15.18%	12.98%	11.02%	0.00%
51	8.57%	7.33%	6.22%	0.00%
58	8.52%	7.29%	6.19%	0.00%
50	5.22%	4.46%	3.79%	0.00%
63	3.24%	2.77%	2.35%	0.00%
Others	1.50%	1.28%	1.09%	0.00%
52	1.07%	0.91%	0.77%	0.00%
68	1.02%	0.87%	0.74%	0.00%
69	0.45%	0.39%	0.33%	0.00%
64	0.44%	0.38%	0.32%	0.00%
19	0.32%	0.27%	0.23%	0.00%
61	0.26%	0.22%	0.19%	0.00%
70	0.26%	0.22%	0.19%	0.00%
72	0.11%	0.09%	0.08%	0.00%
73	0.10%	0.08%	0.07%	0.00%
49	0.09%	0.08%	0.07%	0.00%
74	0.09%	0.08%	0.07%	0.00%
16	0.09%	0.08%	0.06%	0.00%
24	0.08%	0.07%	0.06%	0.00%

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Table 2. (continued)

Species ID	**Control**	**Warming**	**Warming plus**	***Bidens* only**
66	0.03%	0.02%	0.02%	0.00%
46	0.01%	0.01%	0.01%	0.00%
3	0.01%	0.01%	0.01%	0.00%
53	0.01%	0.01%	0.00%	0.00%
75	0.01%	0.01%	0.00%	0.00%
37	0.00%	0.00%	0.00%	0.00%

Others refers to unidentified pollen by morphological traits.

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Table 3. Results of the ANOVAs for bee performance, plant flowering onset and bee larval growth.

Dependent variable	Explanatory variables	χ^2	df	*p* value
Beehive temperature	Temperature treatment	146.93	1	**<0.001**
Number of foragers	Time	40.30	1	**<0.001**
	$Time^2$	4.21	1	**0.040**
	$Time^3$	0.44	1	0.507
	Temperature treatment	0.08	1	0.783
	Ambient temperature	0.72	1	0.397
	Colony size	93.11	1	**<0.001**
	$Time^2$ × Temperature treatment	4.17	1	**0.041**
	$Time^3$ × Temperature treatment	3.98	1	**0.046**
Pollen weight	Time	33.31	1	**<0.001**
	$Time^2$	14.60	1	**<0.001**
	$Time^3$	9.69	1	**0.002**
	Temperature treatment	3.69	1	0.055
	Ambient temperature	4.36	1	**0.037**
	Colony size	9.58	1	**0.002**
	Time × Temperature treatment	4.63	1	**0.031**
Foraging efficiency	Time	11.16	1	**<0.001**
	Temperature treatment	0.27	1	0.603
	Ambient temperature	0.57	1	0.448
Number of flowers	Time	54.15	1	**<0.001**
	$Time^2$	87.99	1	**<0.001**

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Table 3. (continued)

Dependent variable	Explanatory variables	χ^2	df	*p* value
	Temperature treatment	28.84	1	**<0.001**
	$Time^2$ × Temperature treatment	14.44	1	**<0.001**
Larval growth efficiency	Pollen composition	19.46	3	**<0.001**

p values <0.05 are highlighted in bold.

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Table 4. Tukey post-hoc comparisons in the bee larval growth efficiency of different treatments. Note that the growth efficiencies are similar among the warming, warming plus and *B. pilosa*-only treatments, but they are all higher than the efficiency in the control group.

Contrast	**Estimate**	**z**	***p***
Control - Warming	-0.10	-3.90	**<0.001**
Control - Warming plus	-0.07	-2.98	**0.016**
Control - *B. pilosa* only	-0.10	-3.74	**0.001**
Warming - Warming plus	0.03	1.15	0.657
Warming - *B. pilosa* only	0.003	0.11	1.000
Warming plus - *B. pilosa* only	-0.03	-1.02	0.737

p values <0.05 are highlighted in bold.

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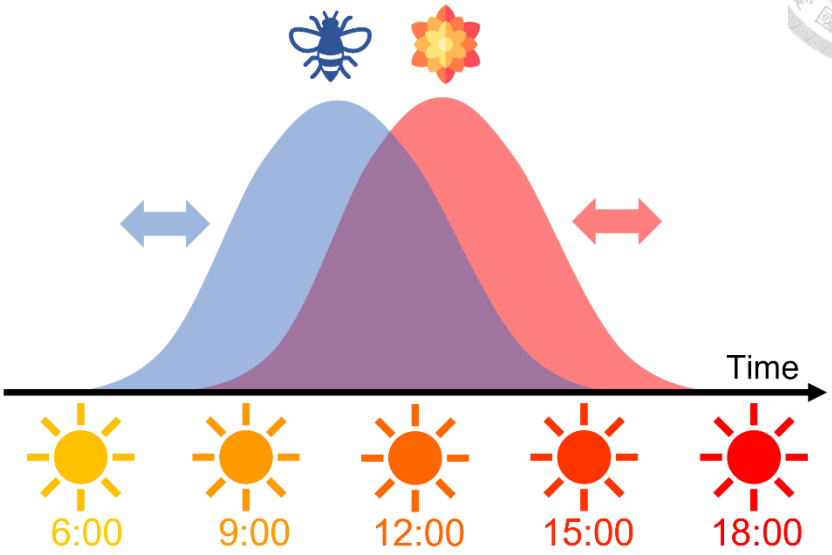

Figure 1. A schematic representation demonstrating the impact of warming on plant pollinator interactions at a shorter temporal scale. For example, elevated temperatures can potentially cause a change in pollinator foraging behavior and/or change in plant flowering onset, resulting in a mismatch of plant-pollinator interactions.

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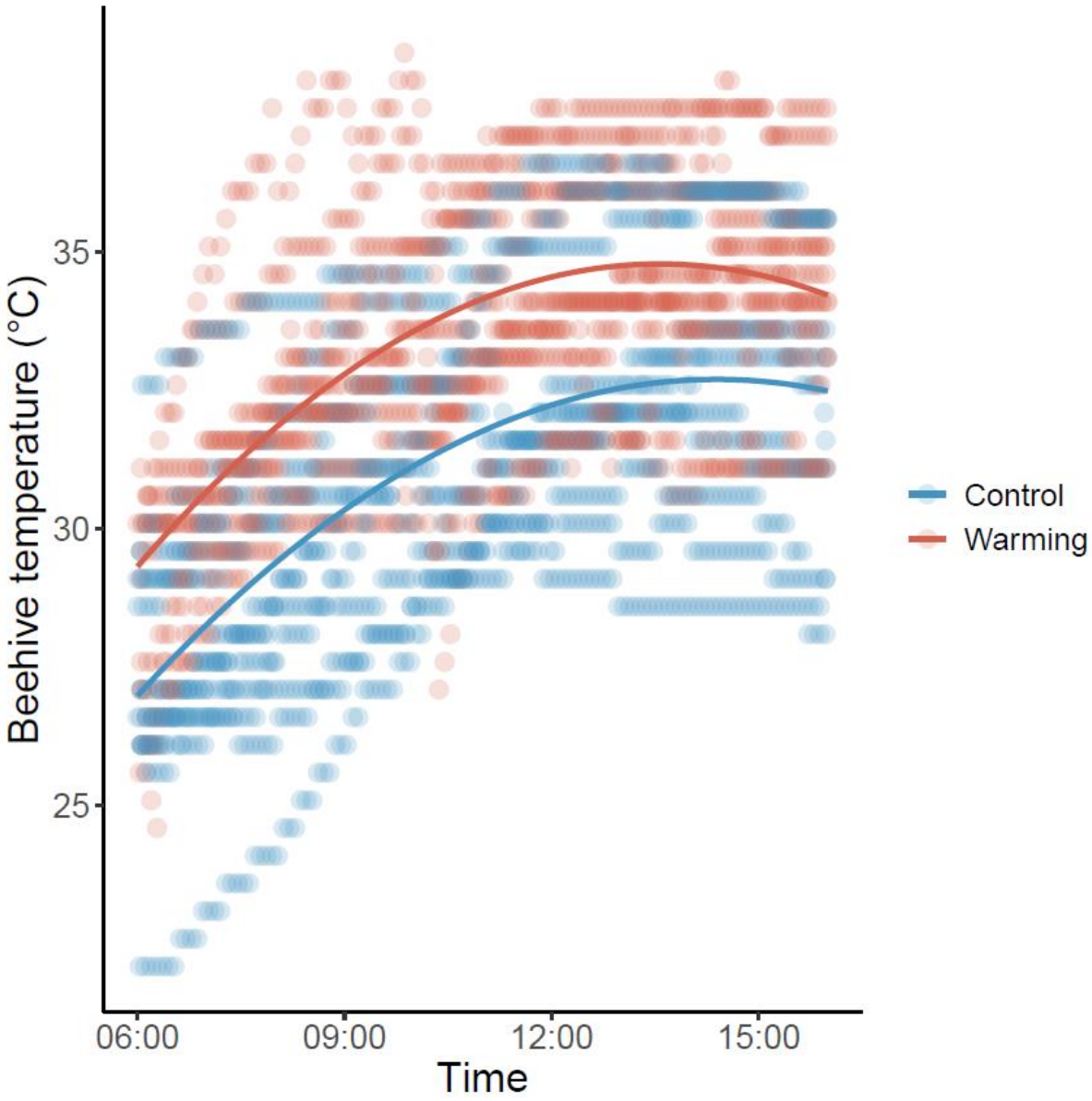

Figure 2. The effect of warming treatment on beehive temperature along with the time of day. Lines are predicted relationships from GLMMs. The beehive temperature of the warming treatment is on average 2.4℃ higher than that of the control group, based on a 25-day temperature record.

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Figure 3. Experimental setup of the beehive, with a camera set above to record the number of foragers

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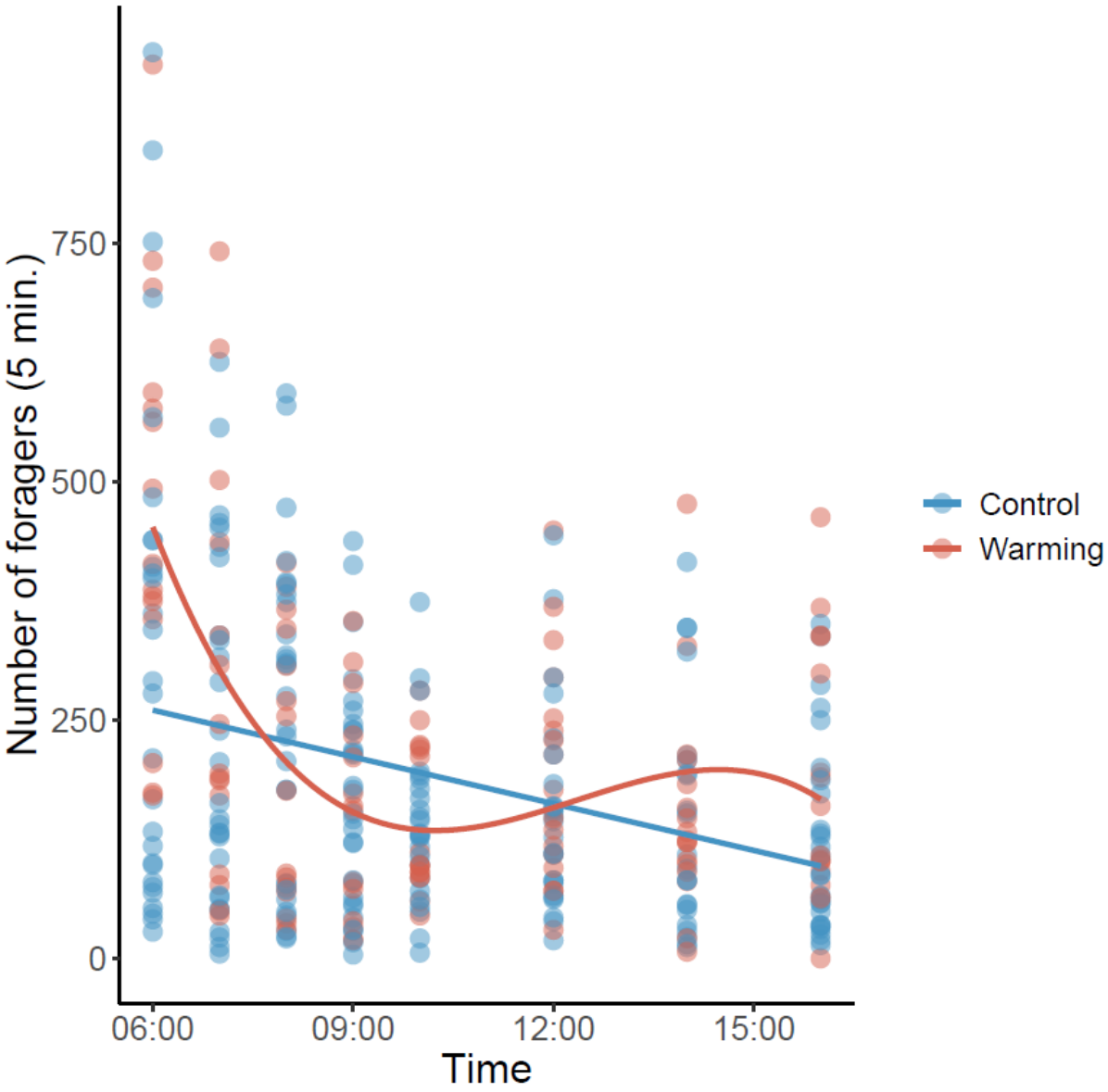

Figure 4. The effect of temperature treatment on the number of foragers going out at different times of day. Warming treatment at 6:00 significantly increased foragers going out ($\chi^2 = 10.12$, df = 1, $p = 0.001$). Lines are predicted relationships from GLMMs.

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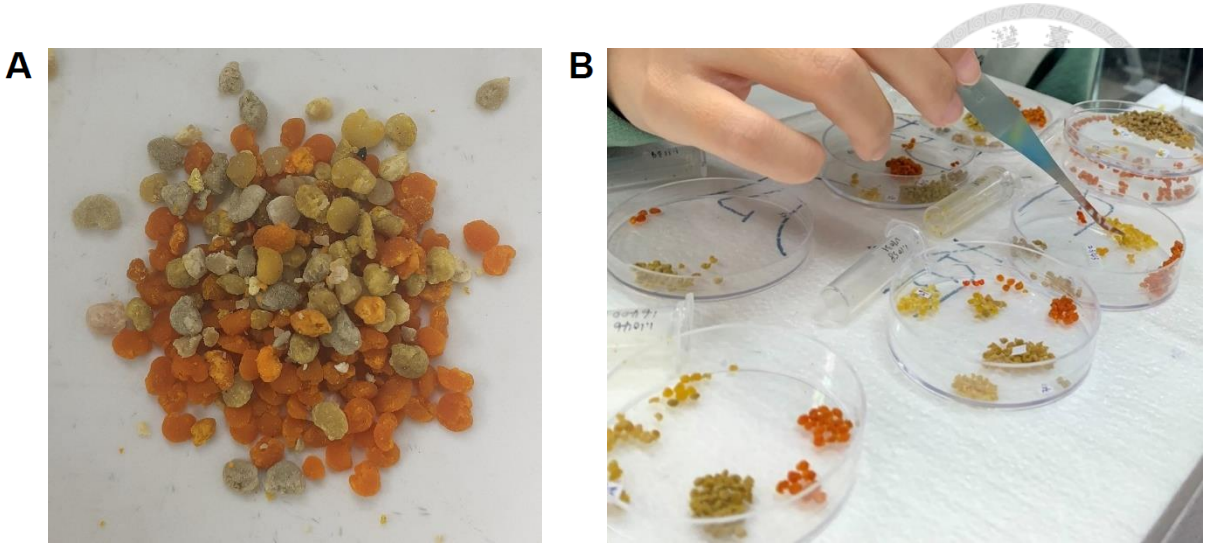

Figure 5. Bee pollen collection and visual classification. An example of bee pollen collected from a beehive (A), with the bee pollen being classified according to their external morphology and color traits for each pollen collection event (B).

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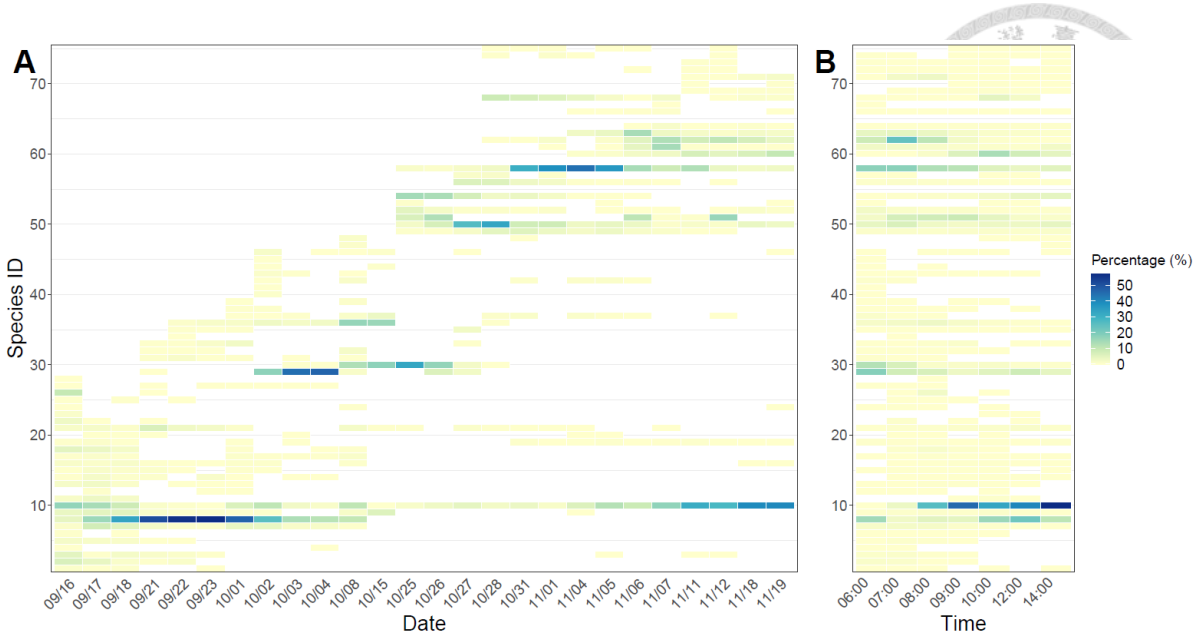

Figure 6. Temporal variations in bee pollen composition during the field warming experiment. Bee pollen abundance of different plant species grouped by different days (A) and by a time of day from 6:00 to 16:00 (B). The most abundant species was *Bidens pilosa* (species ID: 10), followed by *Koelreuteria elegans* (species ID: 8), and then by *Bauhinia* x *blakeana* (species ID: 58), with a percentage of 28.0%, 12.3% and 9.8%, respectively.

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Figure 7. Removal of the disc flowers or their stamens upon anther dehiscence using a pair of tweezers in the laboratory warming experiment on flowering onset.

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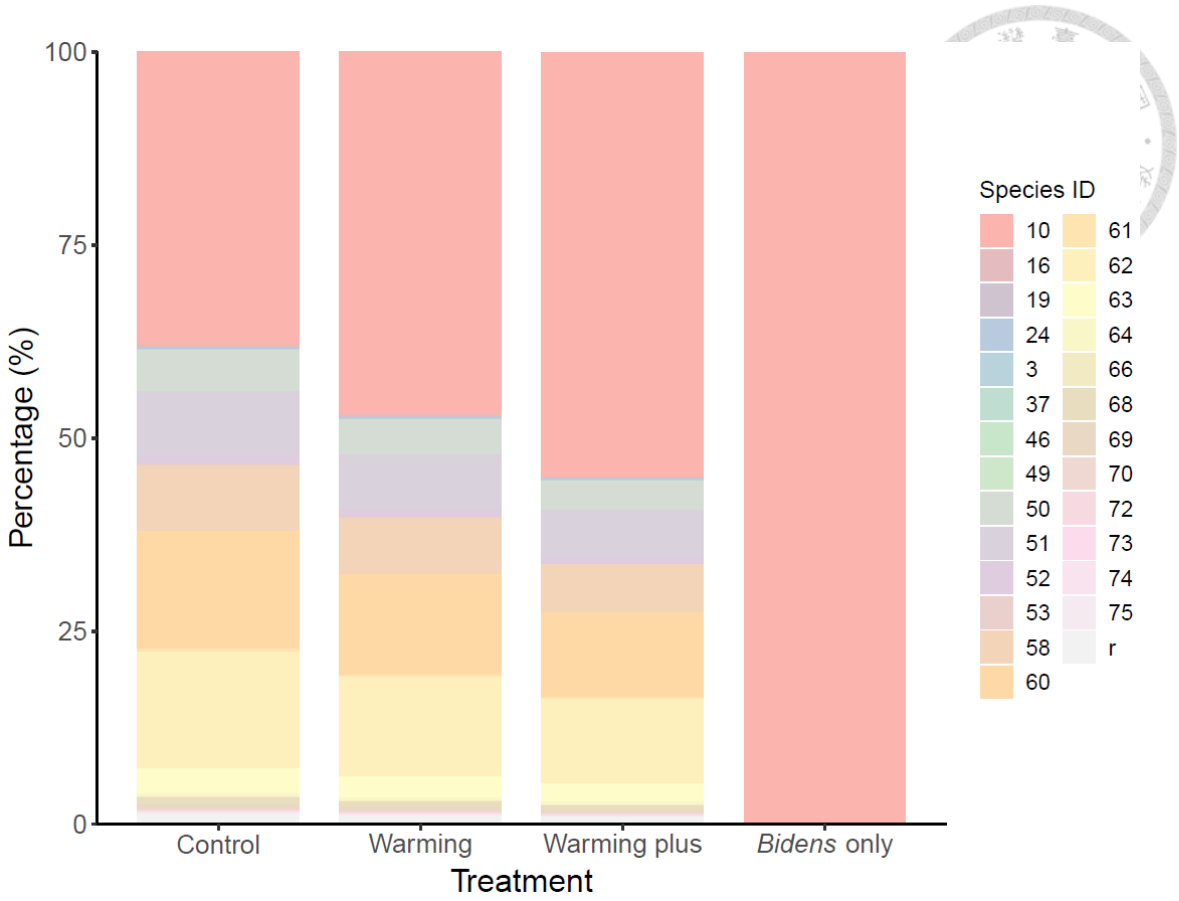

Figure 8. The experimental composition of bee pollen used in the four treatments in the larval feeding experiment. Different colors represent pollen from different plant species (see also Table 2).

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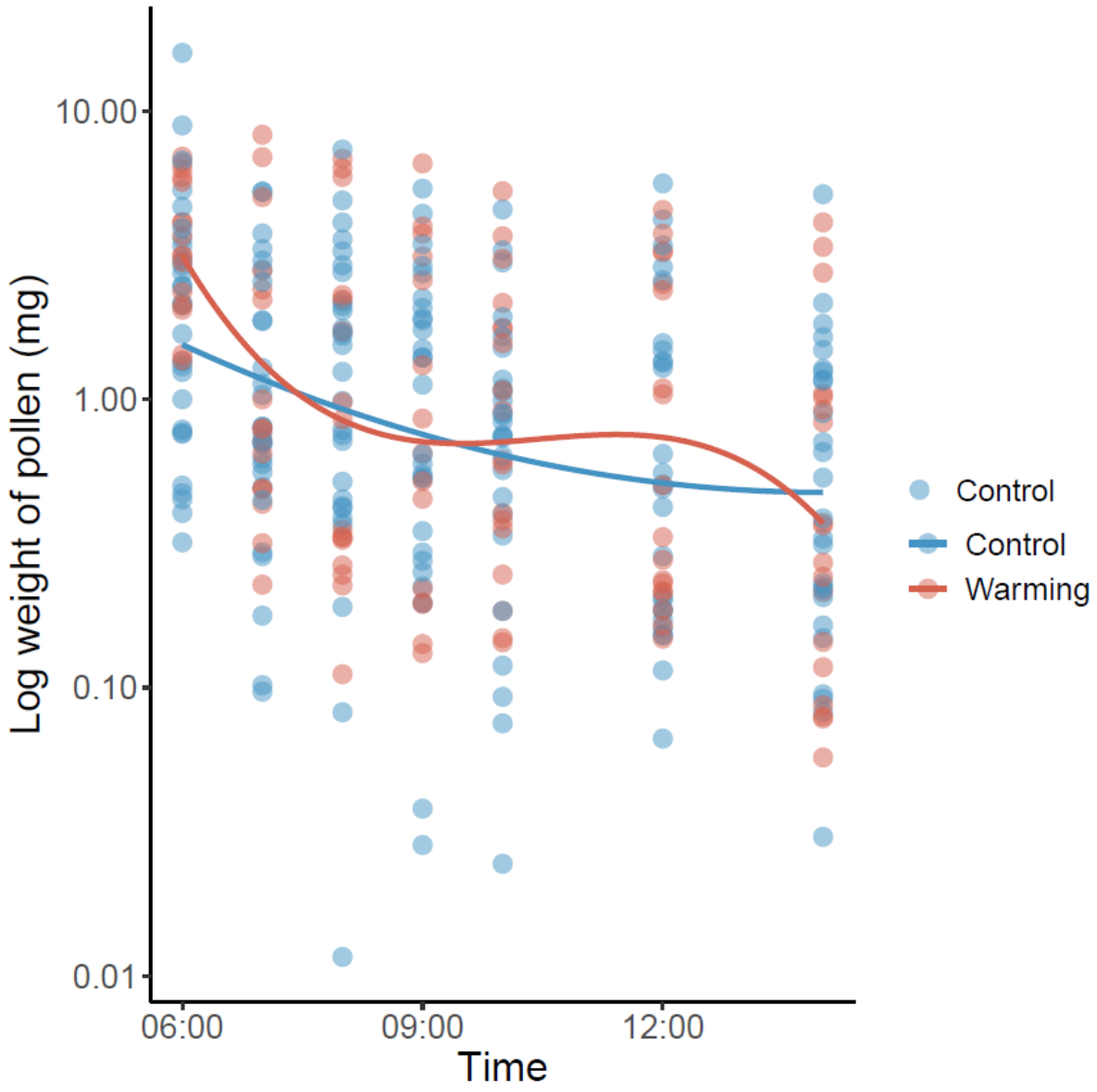

Figure 9. The effect of temperature treatment on the weight of bee pollen at different times of day. Warming treatment at 6:00 significantly increased pollen weight (χ^2 = 5.06, df = 1, p = 0.024). Pollen weight was standardized to the values of one-hour period since pollen was collected once every two hours between 10:00 and16:00. Lines are predicted relationships from GLMMs.

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Figure 10. Feeding larvae with the semi-artificial diet. A side of hive foundation with sufficient number of eggs was chosen, and was brought to the laboratory when the larvae reached 3rd instar. All larvae were kept and fed inside the hive foundation during the experiment under climate-controlled environments.

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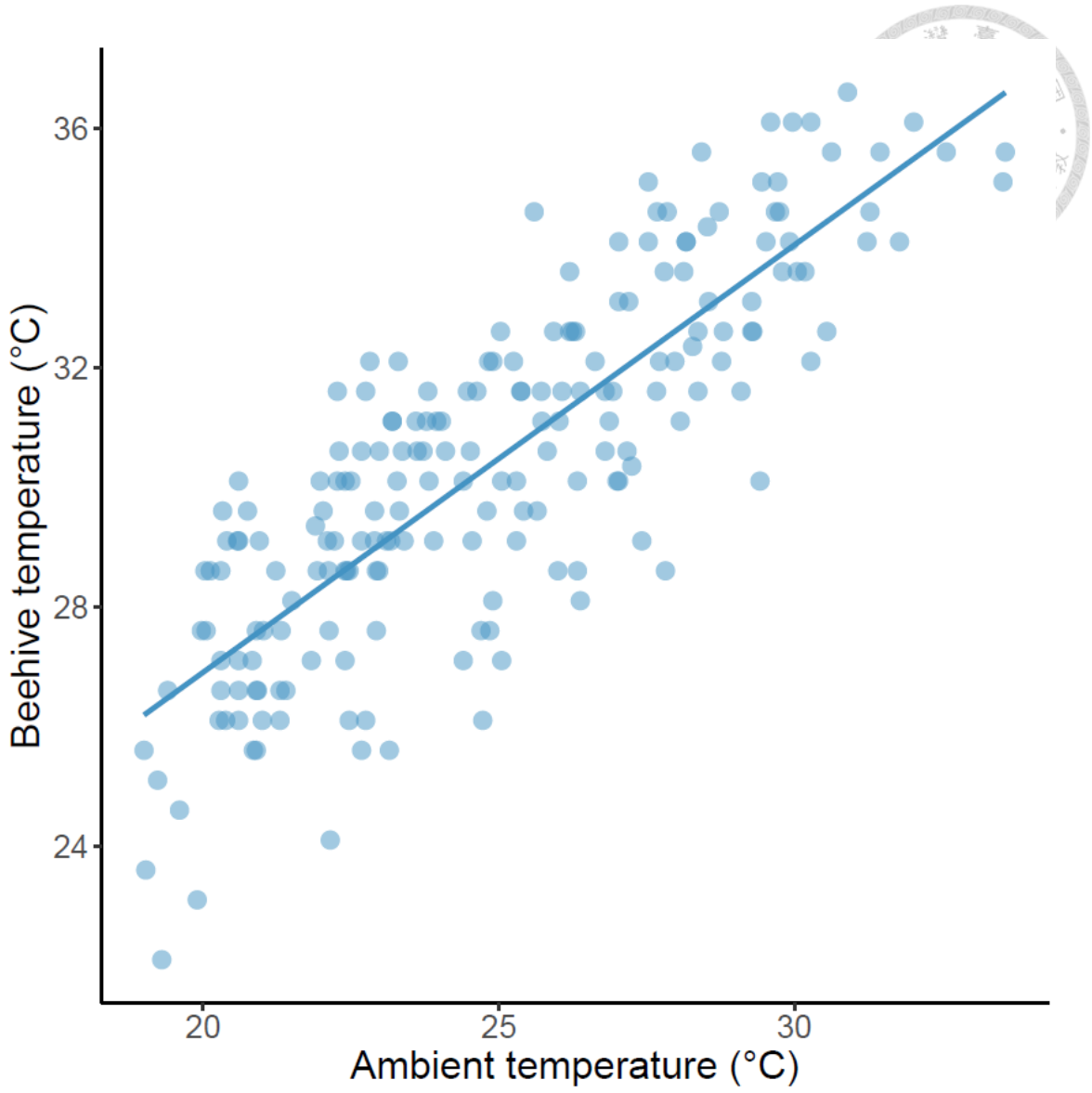

Figure 11. The ambient temperature outside the beehives positively predicted the temperature inside the beehives (Pearson's correlation; $r = 0.83$, $p < 0.001$).

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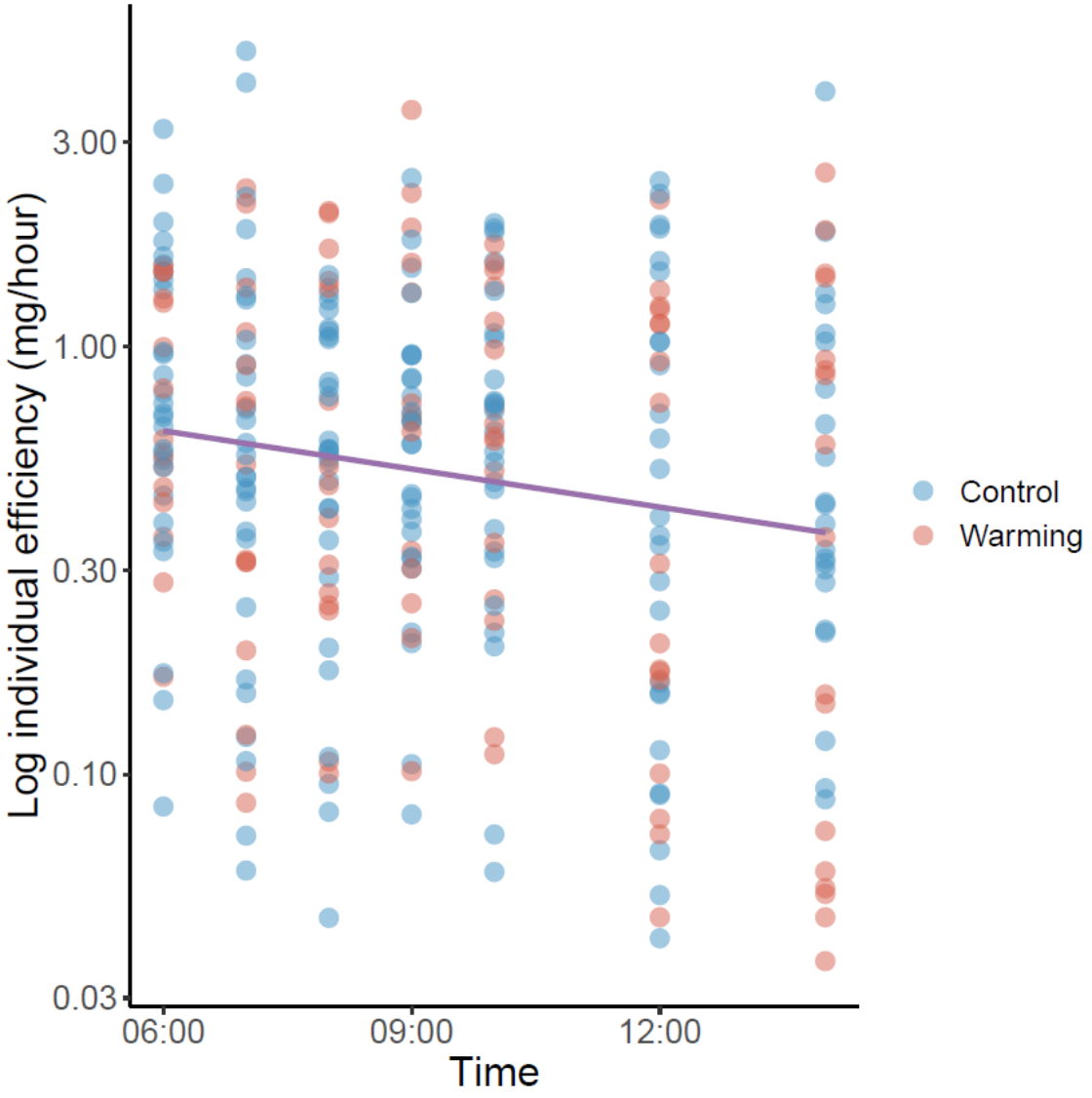

Figure 12. The effect of temperature treatment on individual foraging efficiency at different times of day. The individual foraging efficiency was highest in the early morning and decreased significantly over time (χ^2 = 11.16, df = 1, p < 0.001), with no difference between temperature treatment (χ^2 = 0.27, df = 1, p = 0.603). Lines are predicted relationships from GLMMs.

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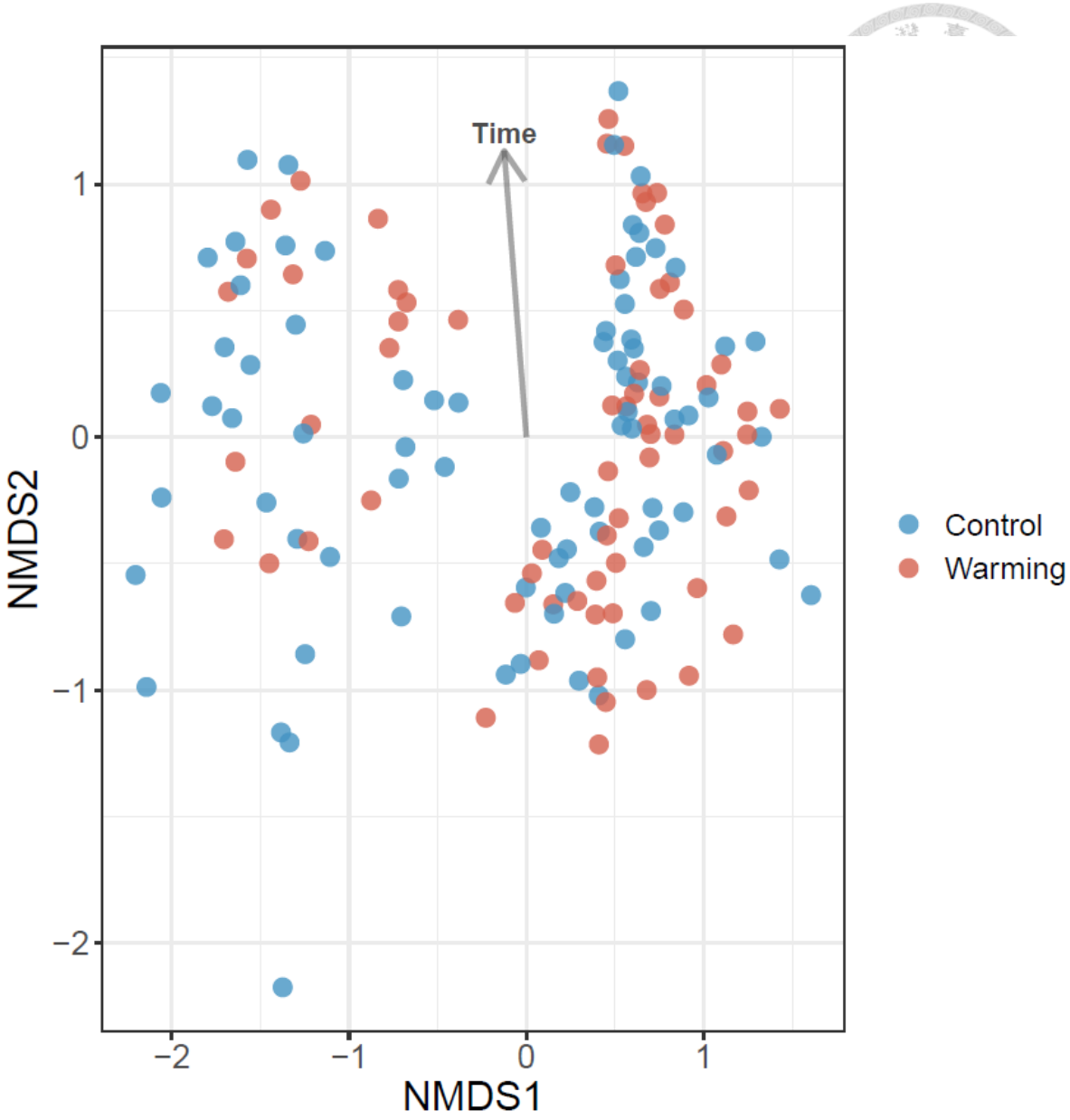

Figure 13. Non-metric multidimensional scaling (NMDS) plot of bee pollen composition. Composition of bee pollen varied over time (F = 5.71, p = 0.001, n = 146) but not with warming treatment.

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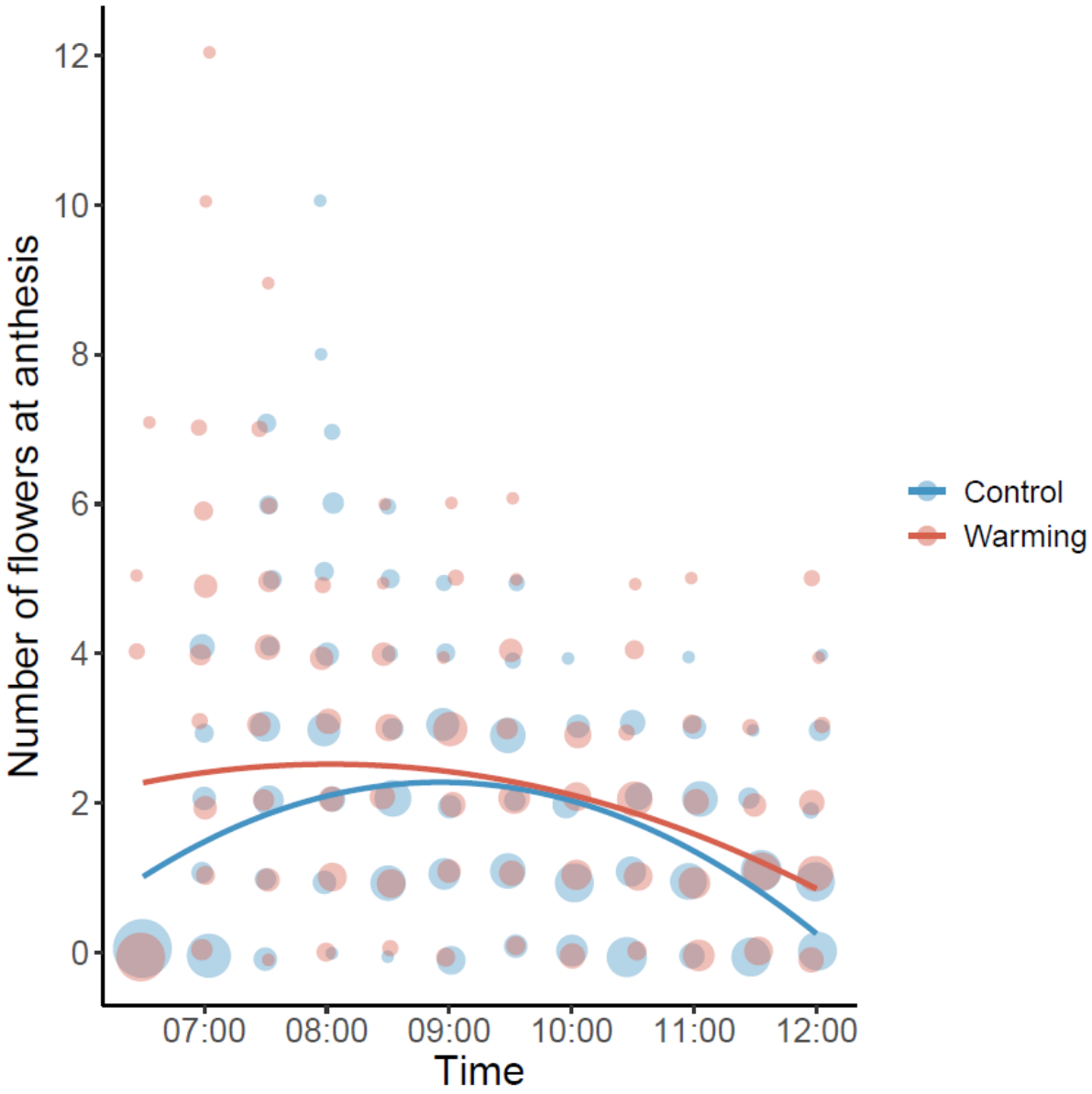

Figure 14. The effect of temperature treatment on the onset of flowers with dehiscent anther. Flowering onset time peaked at 9:00 and 8:00 under control (25/20℃) and warming treatments (30/25℃), respectively. Point size represents the total number of flowers at flowering stage at the certain time. Lines are predicted relationships from GLMMs.

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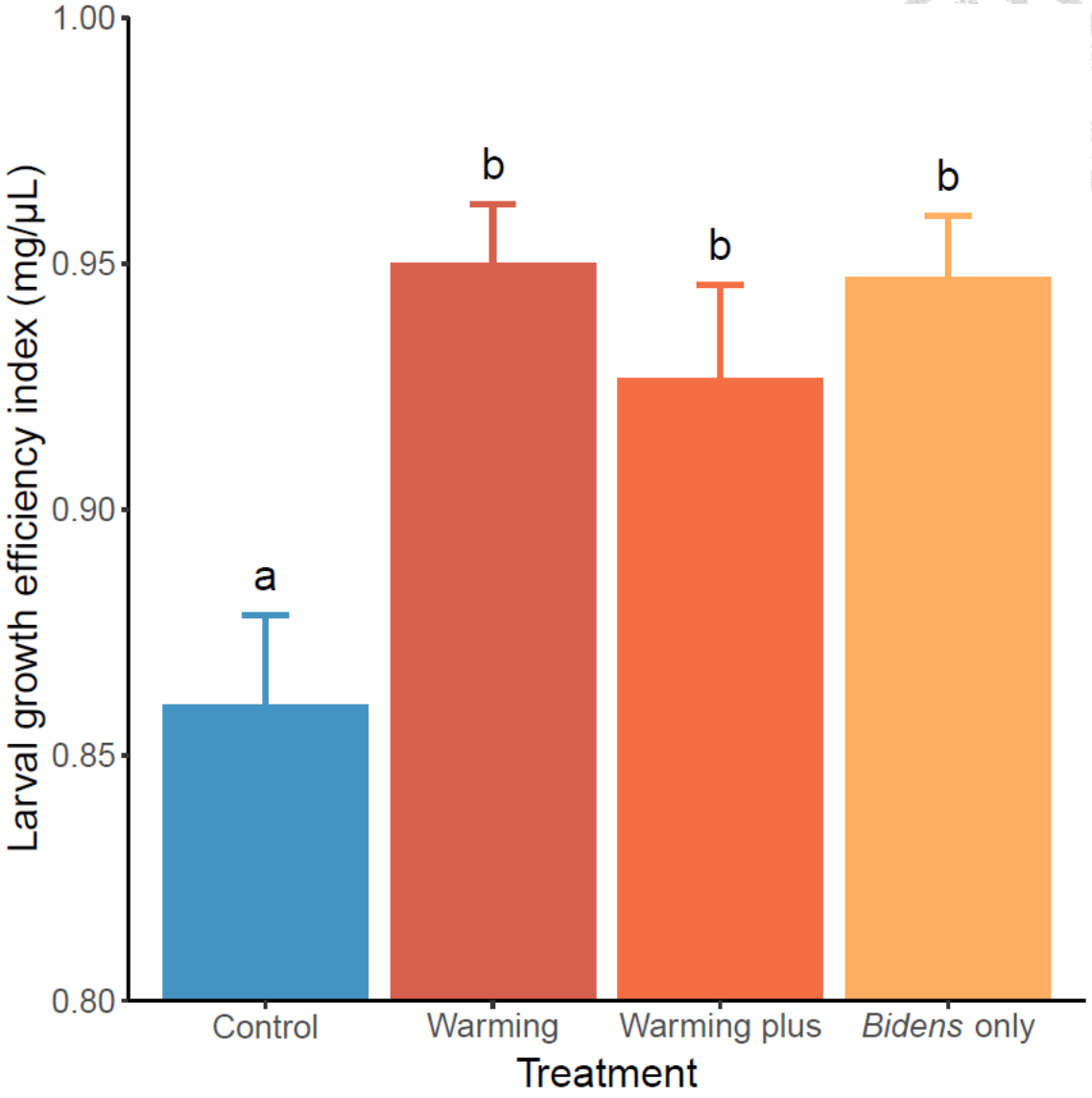

Figure 15. Bee larval growth efficiency (mean ± SE) in the four treatments (the control, warming, warming plus and *Bidens pilosa*-only treatments) of larval feeding experiment.

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