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Warming and soil water availability affect plant–flower visitor interactions for *Stylosanthes capitata*, a tropical forage legume

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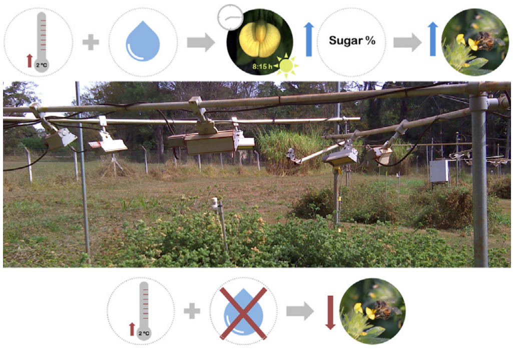
HIGHLIGHTS

- Warming and drought studies on tropical plant-insect interactions are rare.
- Warming increased sugar concentration in nectar of *S. capitata*.
- Warming accelerated flower opening affecting plant-flower visitor interactions.
- Water deficiency and warming negatively affect the number of floral visitors.

GRAPHICAL ABSTRACT



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ABSTRACT

The reproductive success of a zoophilous plant species depends on biological interaction with pollinators, which involves both the provision and exploitation of flower resources. Currently, there is little information about how future climate change scenarios will impact interactions between plants and their flower visitors in the tropics. This study analyzes the effects of warming and two soil water conditions on interactions between the tropical forage legume species *Stylosanthes capitata* and its floral visitors during the flowering period. We used a temperature-free air-controlled enhancement (T-FACE) facility to simulate future warming scenarios by increasing canopy temperature. The tested treatments were: irrigated and ambient canopy temperature (*Control*); non-irrigated and ambient canopy temperature (*wS*); irrigated and elevated canopy temperature (*eT*, + 2 °C above ambient canopy temperature); and non-irrigated and elevated canopy temperature (*wSeT*). The effects of treatments on the time of flower opening and closing, sugar concentration in the nectar, and plant–flower visitor interactions were assessed. In the warmed treatments, *S. capitata* flower opening occurred ~45 min earlier compared to non-warmed treatments, and flowers remained opened for only ~3 h. Further, the sugar concentration in the nectar from *eT* was 39% higher than in the *Control*. The effects of warming on floral biology and flower resource production in *S. capitata* had an impact on the plant–floral visitor relationships with

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the bees *Apis mellifera* and *Paratrigona lineata*, the most abundant potential pollinating floral visitors, and the butterfly visitor *Hemiargus hanno*. Additionally, around noon, the interactive and additive effects of the combined *wS* and *eT* treatments decreased insect visiting frequency. These results suggest that warming and soil water deficiency could affect flower–visitor interactions and thus the reproductive success of *S. capitata* in tropical belts.

1. Introduction

Anthropogenic emissions of greenhouse gases (GHG) such as CO_2, CH_4, and N_2O from the use of fossil fuels, agriculture, livestock production, and land use changes have resulted in an increase in the global temperature of ~1 °C above pre-industrial levels (IPCC, 2021). As a result of continued anthropogenic GHG emissions, climate models predict several future climate scenarios, one of which is a 1.5 to 2 °C increase in average global temperature by the end of the 21st century (IPCC, 2014, 2018). Along with increased temperatures, more frequent and intense drought, heat waves, and flooding are also expected (Wu et al., 2011; Folland et al., 2018; IPCC, 2014, 2018, 2021).

The percentage of animal-pollinated species increases from 78% in the temperate belt to 94% in the tropics (Ollerton et al., 2011), where native and cultivated plants are pollinated by more than 20,000 bees and other insects, and vertebrates (Patricio-Roberto and Campos, 2014; IPBES, 2016). However, the individual and combined effects of global warming and drought threaten the adaptive capacity of ecosystems which can have significant ecological and economic impacts on the world's plant and animal species (Ockendon et al., 2014; IPCC, 2014, 2018; Malhi et al., 2020; Román-Palacios and Wiens, 2020; Pörtner et al., 2021). Climate change may disrupt interspecific interactions between pollinators and plants (Memmott et al., 2007; Hegland et al., 2009; DeLucia et al., 2012; Ockendon et al., 2014; Alzate-Marin et al., 2021a,b), affecting human livelihoods, food security, and public health, especially among marginalized populations (Kjøhl et al., 2011; Pörtner et al., 2021).

Temperature affects the timing of both plant flowering and pollinator activity (Scaven and Rafferty, 2013). Insects and plants may respond differently to changing temperatures, producing phenological mismatches that can affect plants, due to a reduction in insect visitation and pollen deposition, as well as pollinators through reduced access to food resources (Hegland et al., 2009). The reproductive success of plants is associated with flower opening/closing strategies since these strategies affect pollen removal, pollination, and the provision of resources by the plant (van Doorn and van Meeteren, 2003; Baude et al., 2016). Also, the timing of nectar production determines when flower visitation occurs, influencing the reproductive success of plants (Biella et al., 2021). The effects of warming on early floral opening (Alzate-Marin et al., 2021a) and high rate of early photosynthesis (Habermann et al., 2019, 2021) have been observed for *Stylosanthes capitata*. Temperature-induced early floral opening is probably associated with early morning photosynthesis and endogenous auxin activity (Ke et al., 2018). Variations in temperature and water availability also have an impact on nectar secretion and sugar content (Petanidou and Smets, 1996; Descamps et al., 2018, 2021), affecting the behavior of flower visitors. Furthermore, warmer flowers may be more attractive to pollinators, as changes in color help to improve their detectability by insects, while bees tend to prefer warm nectar (Norgate et al., 2010). This preference is associated with the lower viscosity of warmed nectar, which allows for faster feeding, providing the energy needed for flight (Atamian et al., 2016; Dyer et al., 2006; Afik and Shafir, 2007; Nicolson et al., 2013). However, sucrose concentration takes precedence over temperature, so bees tend to choose the sweetest source, even if the less sweet source is warmer (Whitney et al., 2008). In extreme situations, plants subjected to heat and water stress can begin flowering without producing nectar, limit the number of flowers and the proportion of flowers with nectar, or flower earlier as a drought mitigation strategy (Petanidou and Smets, 1996; Ivey and Carr, 2012; Phillips et al., 2018). The consequences of such changes in floral characteristics can lead to a reduction or even suppression in the number of visits by pollinating insects, thus compromising the intricate interactions between these organisms (Hoover et al., 2012; Scaven and Rafferty, 2013).

Although many studies have examined the effects of elevated temperature, drought, and water stress on crop growth, development, and yield (Prasad et al., 2008), the impacts of these abiotic stresses on pollination are less well understood (Kjøhl et al., 2011; Mueller et al., 2020). Further, studies evaluating the effects of climate change on plant–insect interactions in the tropics are scarce (Deutsch et al., 2008; Descamps et al., 2021). Here, we studied the effects of warming (an increase of 2 °C above ambient canopy temperature) and water deficit on plant–flower visitor interactions for *Stylosanthes capitata* Vogel, a sub-shrub, leguminous species of the Fabaceae family that is native to South America. The species is ecologically and economically important as forage as it can be grown in acidic and low-fertility soils in savanna regions (Alzate-Marin et al., 2020; Cook et al., 2020; Schultze-Kraft et al., 2020). *S. capitata* depends on pollinators to produce self-fertilized and outcrossed seeds since it has a mixed mating system with outcrossing rates ranging from 12% to 53% (Miles, 1983; Santos-Garcia et al., 2011; Alzate-Marin et al., 2021a).

Previous studies have shown that plants subjected to heat and water stress can limit their floral resources as a drought mitigation strategy (Petanidou and Smets, 1996; Ivey and Carr, 2012; Phillips et al., 2018; Hoover et al., 2012; Scaven and Rafferty, 2013). We hypothesize that the interaction of warming and water deficit will decrease floral visitors and pollinator attraction to *S. capitata* due to the adverse effects of these stresses on nectar and sugar production and reproductive investment. A previous study (Alzate-Marin et al., 2021a) showed that warming, alone or with elevated CO_2, stimulates early floral opening and closing in *S. capitata* plants. Thus, we further hypothesize that the exposure of *S. capitata* plants to warming and its interaction with water deficiency will affect the timing of flower opening and closing, creating a mismatch for floral visitors between warmed and non-warmed treatments. To test our hypotheses, we analyzed the separate and combined effects of warming (+2 °C) and water deficit on *S. capitata* in terms of: 1) flower opening and closure; 2) sugar concentration in the nectar; and 3) potential floral visitors and pollinators.

2. Methods

2.1. Experimental area and treatments

This study was conducted under field conditions at the University of São Paulo, Ribeirão Preto Campus (São Paulo State, Brazil; 21°10′08.63 ″S, 47°51′50.47″ W; elevation: 578 m) (Google Earth Pro, 2020). The climate in Ribeirão Preto is classified as Aw (Tropical wet and dry climate) based on the Köppen classification (Dubreuil et al., 2019), with dry winters and hot, rainy summers. From 2010 to 2018, the average annual temperature was 23 °C and rainfall was 1238 mm (CIIAGRO, 2021).

S. capitata plants were grown in 4 × 4 m plots and submitted to four treatments, each with three repetitions: *Control* – irrigation and ambient canopy temperature (*Tcanopy*); *wS (water stress)* – non-irrigation and ambient *Tcanopy*; *eT* – irrigation and elevated *Tcanopy* (+2 °C above ambient); and *wSeT* – non-irrigation and elevated *Tcanopy*.

The experiments were installed using a T-FACE facility (Kimball et al., 2008) (Fig. S1a–c). Throughout the entire plant growth cycle, the *Tcanopy* for *S. capitata* plants in warmed plots (*eT* and *wSeT*) was maintained at 2 °C above the *Tcanopy* of non-warmed (*Control* and *wS*) plots using six 750 W infrared heaters, model FTE-750-240 V (Mor Electric Heating, Comstock Park, MI, USA), in each plot. The heaters were installed in aluminum reflectors, model Salamander ALEX (Mor Electric Heating, Comstock Park, MI, USA), and placed at 0.8 m above the plant canopy in a hexagonal arrangement (Fig. S1b). The elevated temperature of warmed plots was maintained using a proportional-integrative-derivative (PID) control algorithm installed in a datalogger, model CR1000, with AM25T multiplexors (Campbell Scientific, Logan, UT, USA) (Fig. S1c). In each plot, infrared radiometers, model SI-1H1-L20 (Apogee Instruments, Logan, UT, USA), monitored the *Tcanopy*.

In the *Control* plots, the minimum and maximum recorded *Tcanopy* were 8 and 32 °C, respectively, throughout the experimental period. In the warmed plots, the daily average increase in temperature was 1.85 ± 0.20 °C, and the average nightly increase in temperature was 1.95 ± 0.18 °C. During the day, the *Tcanopy* of warmed plots was probably influenced by wind and high evapotranspiration rates, despite the equipment set-point to 2 °C above ambient temperature. However, the T-FACE system was able to raise the temperatures of the heated plots by the target amounts most of the time throughout the experiment. About 80% of observations of canopy temperature were within 0.2 °C of the desired temperature increase (2 °C) (Kimball et al., 2008).

An automatic drip irrigation system was used to control soil water content (SWC) of treatments (Fig. S1 d–g). The SWC and soil temperature were monitored respectively by Theta probe ML2X and ST1 sensors (Delta-T Devices Ltd., Burwell, Cambridge, UK), placed in the center of each plot at a depth of 10 cm, and connected to a DL2e datalogger (Delta-T Devices Ltd., Burwell, Cambridge, UK). From sowing to the end of the experiment, the SWC of *Control* and *eT* treatments were maintained continuously near field capacity. However, in *wS* and *wSeT* treatments, irrigation was suspended for 40 days after plant establishment. Once the irrigation was stopped, the non-irrigated treatments experienced a gradual decrease in SWC. During the flowering period, the average SWC of *Control* and *eT* were 0.50 and 0.53 $m^3\ m^{-3}$, respectively, while the average SWC in *wS* and *wSeT* were 0.23 and 0.24 $m^3\ m^{-3}$, respectively. The minimum value of SWC registered in non-irrigated plots was 0.16 $m^3\ m^{-3}$ at the end of the stress period when the net photosynthetic rate was close to zero, as measured by a portable infrared gas analyzer (LCPro, ADC BioScientific Ltd., Hoddesdon, UK). A more detailed description of the T-FACE facility and irrigation system is available in Habermann et al. (2021).

2.2. Collection of flower visitors and monitoring of floral opening and closure

During seven weeks of the *S. capitata* flowering period, from April 24 to June 14, 2018, flower visitors were monitored in all three replicate plots across all treatments. One plot (replication) of each of the four treatments was assessed daily, for a total of three days of evaluation per week. Each plot was monitored for 10 min six times per day between 08:00 and 13:00 h, with a rest interval of one hour between monitoring. The insects were collected with a hand net, euthanized with ethyl acetate, and pinned. All collected insects were quantified and identified at the level of order (Coleoptera, Diptera, Hymenoptera, and Lepidoptera), with bees identified to the family, tribe, and species level. To determine the pollinating potential of bees, we evaluated the deposition of *S. capitata* pollen grains on their bodies. For pollen identification, samples of pollen grains from *S. capitata* flowers and pollen from bees' bodies (removed with adhesive tape) were analyzed simultaneously using an optical stereomicroscope model Discovery. V12 (Carl Zeiss Microscopy, Jena, Germany), following RCPol (2021) and Alzate-Marin et al. (2021b). We also conducted similar analyses on some specimens of the most frequent butterfly species that visited the flowers.

Alongside the assessment of floral visitors, we monitored the floral opening and closure times in each plot for four weeks (three days/week), in the last week of April, the second and third week of May, and the second week of June 2018. We also conducted a quick survey to record floral temperatures using an infrared thermal imaging camera model FLIR T400 (FLIR Systems, Wilsonville, Oregon, USA).

2.3. Analysis of sugar concentration in the nectar

To measure sugar concentration in the *S. capitata* nectar, we used an Eclipse Professional Optical Refractometer model 45–81 Low Volume (Nectar <1-micro-litre), with a range of 0–50, and a 0.5 scale division (Bellingham + Stanley, Kent, UK). However, due to the small amount of nectar in each flower, we experimented with different standard sugar concentrations and selected a 10% sugar solution diluted in autoclaved ultrapure water (Direct-Q, Merck KGaA, Darmstadt, Germany) for the analysis. Using a micropipette, we placed 2.5 μL of the 10% standard sugar solution in each nectary, mixing the solution carefully with the flower's nectar content. The nectar solution was then collected, and sugar concentration was measured with a refractometer. We considered the percentage of sugar measured above the standard amount (10%) to be the sugar concentration of nectar.

The day before nectar was collected, we bagged the inflorescences in the afternoon to avoid loss by pollinators. We analyzed five flowers per plot in two plots of each treatment (40 flowers) on two dates of the same week (22 and 25 May 2018) during the flowering period. Five flowers of each treatment were sampled for dissection, nectary visual observation, and photography. Flower images were captured with a Leica ICC50 video camera installed in a Leica DM500 light microscope connected to a computer using the Leica IM50 digital image analysis system Image-pro® Plus 6.0 software.

2.4. Statistical analysis

The data were tested for normal distribution using the Anderson-Darling test (Nist/Sematech, 2003) in the PAST 4.03 software (Hammer et al., 2001). Data that did not show normal distribution were transformed using the Box-Cox method in the same software. For the flower opening and closure statistical analyses, hours were converted to seconds. The results are presented in hours.

We used analysis of variance (ANOVA) in a factorial scheme with three factors and three repetitions to evaluate flower opening and closing time, visitation by the bees *Apis mellifera* and *Paratrigona lineata*, and sugar concentration in nectar. Two factors were standard for all analyses: *wS* (with and without irrigation) and *eT* (with and without warming). The third factor was time: for flower opening and closing, the time factor was the week; for visitation by the bees *A. mellifera* and *P. lineata*, the time factor was the hour; and for nectar sugar concentration the time factor was the day.

For the insect *Tetragonistica angustula*, "other bees", and the butterfly *Hemiargus hanno*, hourly visitor data did not show normal distribution even after data transformation. As the total number of visitors had normal distribution, the ANOVA was performed in a factorial scheme with two factors (*wS* and *eT*) and three repetitions. Analyses were performed with mixed models, in which the factors *wS* and *eT* were considered fixed effects, and hour, day, and week were considered random effects.

When the main effects or interactions between factors were significant, treatment means were compared using the Fisher's LSD post hoc test at 5% probability. All analyses were done using PAST 4.03 (Hammer et al., 2001) and Minitab 18 (2018).

3. Results

3.1. Flower opening and closure

In the warmed plots (*eT* and *wSeT*), *S. capitata* flowers began opening around 08:15, with a canopy temperature (*Tcanopy*) of ~20.5 °C, total solar radiation (TSR) of ~0.23 $kW\ m^{-2}$, relative humidity (RH) of ~85%, and wind speed (WiS) of ~0.14 $m\ s^{-1}$. The closing of flowers in warmed plots occurred at ~11:10, with a *Tcanopy* of ~26 °C, TSR of ~0.47 $kW\ m^{-2}$, RH of ~59%, and WiS of ~1 $m\ s^{-1}$. The flowers in non-warmed plots (*Control* and *wS*) opened approximately 45 min later at ~09:00, with a *Tcanopy* of ~22.5 °C, TSR of ~0.35 $kW\ m^{-2}$, RH of ~71%, and WiS of ~0.60 $m\ s^{-1}$. The *Control* and *wS* flowers closed with a delay of about 80 min at ~12:30, with a *Tcanopy* of ~25 °C, TSR of ~0.45 $kW\ m^{-2}$, RH of ~55%, and WiS of ~1.12 $m\ s^{-1}$ (Table 1). In warmed conditions, the flowers remained open for an average of 35 min less than in the non-warmed treatments, resulting in less time for visitors

Table 1

Analysis of variance for mean time of flower opening and closure of *Stylosanthes capitata* exposed to four treatments: *Control* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*.

Source of variation	Mean time of flower opening ± SE	*P-value*	Mean time of flower closure ± SE	*P-value*
Control[a]	09:01 ± 00:06	–	12:34 ± 00:05	–
wS	09:05 ± 00:06	0.775ns	12:33 ± 00:05	0.810ns
eT	08:15 ± 00:11	0.000**	11:07 ± 00:07	0.000**
wS × *eT*	08:15 ± 00.09	0.723ns	11:11 ± 00.05	0.715ns
Weeks	08:39 ± 00:14	0.001**	11:52 ± 00:24	0.145ns
Week × *wS*	–	0.987ns	–	0.915ns
Week × *eT*	–	0.492ns	–	0.268ns
Week × *wS* × *eT*	–	0.992ns	–	0.944ns

** Significant at 1% probability, ns = Not significant.

[a] The mean and standard error (SE) for *Control* is presented for reference as *Control* is not a source of variation in the analysis of variance.

to find open flowers. A quick survey of floral temperatures with thermal images showed that at ~11:30, the mean flower temperatures of the *Control*, *wS*, *eT*, and *wSeT* treatments were 22.0 ± 0.2, 24.0 ± 0.4, 25.7 ± 0.1, and 23.0 ± 0.2 °C, respectively (Fig. 1).

At the time of flower opening, the ANOVA showed a significant effect of the factors *eT* ($p \leq 0.001$) and week ($p \leq 0.001$) (Table 1). For the time of flower closure, we only observed a significant effect of *eT* ($p \leq 0.001$) (Table 1). The *wS* factor did not affect flower opening and closing time. Further, we did not observe an interaction between factors for either flower opening or closure (Table 1).

In the analyses by week, we observed earlier flower opening in the warmed treatments compared to non-warmed treatments in the first two weeks and for the overall mean time (Fig. 2a). We also observed earlier flower closing in warmed treatments compared to non-warmed treatments in all weeks and considering the overall mean (Fig. 2b).

3.2. Sugar concentration in nectar and visual analysis of the nectary

The ANOVA showed a significant effect of the factor *eT* ($p \leq 0.050$), no effect of *wS*, and no interaction between them (Table 2). Sugar concentration in *S. capitata* nectar increased significantly (39%) under *eT* (1.63 ± 0.01%) compared to the *Control* (1.17 ± 0.13%) (Table 2).

In the analyses of the treatments, we observed significant differences between combined *wSeT* in comparison to *Control* for sugar concentration (Fig. 2c). Since the ANOVA showed neither *wS* factor effects nor interaction between *wS* and *eT*, we suggest that in the combined treatment *wSeT*, the higher level of sugar concentration is due only to *eT*. Nectary images are shown in Fig. S2.

3.3. Floral visitors

We collected a total of 2149 insects, including Coleoptera (n = 5), Diptera (n = 30), Hymenoptera (wasps) (n = 33), Lepidoptera (n = 335), and Hymenoptera (bees) (n = 1746) (Table 3). Bees of the tribes Apini, Meliponini, Augochlorini, Exomalopsini, and Megachilini were considered potential pollinators of *S. capitata* based on individual bee behavior, presence of pollen traces on their bodies, or both. *Apis mellifera* was the most abundant bee species (68%) capable of achieving *S. capitata* pollination by collecting nectar and pollen. Its body size (~13 mm) is compatible with the keel drive mechanism in *S. capitata* flowers. We observed that of the analyzed *A. mellifera* individuals, 77% had *S. capitata* pollen in their tibial corbiculae.

Another frequent visitor identified as a potential *S. capitata* pollinator was *P. lineata* as we observed pollen in the corbiculae (21% of the collected

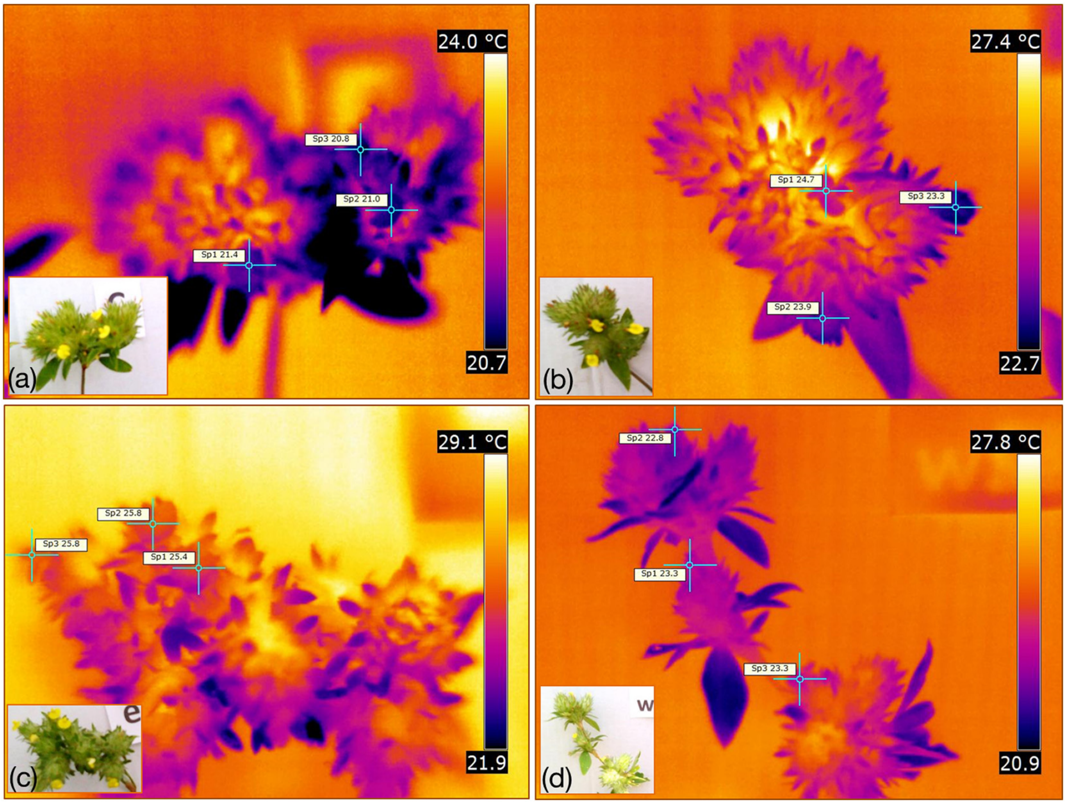

Fig. 1. Thermal images of *Stylosanthes capitata* flowers in the following treatments: (a) *Control*; (b) *wS* – non-irrigated and ambient *Tcanopy*; (c) *eT* – irrigated and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and (d) *wSeT* – non-irrigated and elevated *Tcanopy*. The visible light images of *S. capitata* inflorescences and flowers are shown in the boxes at the bottom left. The pictures were taken using an infrared thermal imaging camera model FLIR T400 (FLIR Systems, Wilsonville, Oregon, USA) on 08/06/2018 at 11:30.

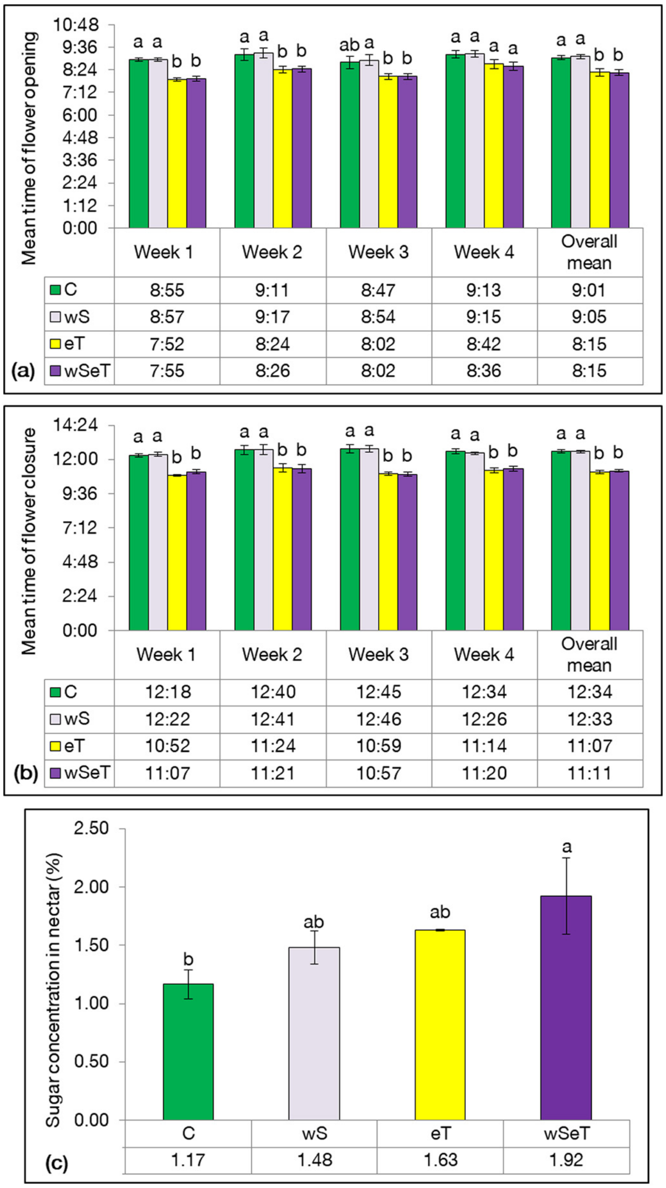

Fig. 2. Mean time of *Stylosanthes capitata* flower opening (a) and closure (b), and sugar concentration in nectar (c) as a function of treatments (*Control* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*). Treatments followed by the same letter do not differ statistically based on the LSD Fisher test at 5%.

bees) and ventral region of the thorax (47%) (Fig. 3a, e). Due to its small body size (~4.5 mm in length), it is difficult to activate the trigger mechanism of the keel in *S. capitata* flowers. Therefore, their behavior may be opportunistic, accessing flowers already opened by *A. mellifera* or other bees. The species of Augochlorini (Fig. 3b), Exomalopsini (Fig. 3c), and Megachilini (Fig. 3d), with body sizes ranging from 8 to 10 mm in length that collected pollen grains attached to the ventral or sternal scopes, were also identified as potential pollinators, supporting the observation by Alzate-Marin et al. (2021a). The butterfly *H. hanno* frequently collected nectar from the *S. capitata* flowers (Fig. 3f); however, we did not identify pollen grains on the bodies of the analyzed specimens.

We analyzed the time of flower visiting by *A. mellifera* (Fig. 4a) and *P. lineata* (Fig. 4b) between 08:00 to 12:00 and 09:00 to 13:00, respectively, when the treatments were most frequently visited by bees. The ANOVA for *A. mellifera* showed significant effects of *wS* ($p \leq 0.001$), *eT* ($p \leq 0.001$), and hour ($p \leq 0.001$), and interaction between *wS* × *eT* ($p \leq 0.041$), and hour × *eT* ($p \leq 0.001$) (Table 4). Therefore, the time of flower visiting depended on the temperature in *wS* and the hour in *eT* (Table 4). The number of *A. mellifera* visits was highest in *eT* treatments between 08:00 and 10:00 (Fig. 4a). At 08:00 in the warmed treatments *eT* and *wSeT*, we observed a significantly greater number of *A. mellifera* visits than the non-warmed treatments *Control* and *wS* (Fig. 4a), regardless of water stress conditions. At 09:00, there were also more visits in the *eT* treatment when compared to the non-warmed treatments. However, at 11:00 and 12:00, we observed lower *A. mellifera* visitor frequency in *wSeT* treatments when compared to *Control* (Fig. 4a) due to the interactive effect of *wS* and *eT* (Table 4).

Table 2
Analysis of variance for the mean percentage (%) of sugar in nectar from *Stylosanthes capitata* flowers exposed to four treatments: *Control* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*.

Source of variation	Mean % of sugar in nectar ± SE	*P-value*
Control[a]	1.17 ± 0.13	–
wS	1.48 ± 0.14	0.159ns
eT	1.63 ± 0.01	0.050*
wS × *eT*	1.92 ± 0.32	0.952ns
Day	1.55 ± 0.15	0.162ns
Day × *wS*	–	0.419ns
Day × *eT*	–	0.864ns
Day × *wS* × *eT*	–	0.468ns

* Significant at 5% probability, ns = Not significant.

[a] The mean and standard error (SE) for *Control* is presented for reference as *Control* is not a source of variation in the analysis of variance.

For the number of *P. lineata* visits, the ANOVA showed significant effects of *wS* ($p \leq 0.003$), *eT* ($p \leq 0.002$), and hour ($p \leq 0.001$), with no interactions between the factors (Table 4). At 09:00, *P. lineata* showed a similar number of visits to *S. capitata* flowers in all treatments. At 10:00, we observed greater *P. lineata* visitor frequency in the *eT* treatment when compared to the *wSeT*. Between 11:00 and 13:00, the additive effect of *wS* and *eT* resulted in fewer *P. lineata* visits in comparison to *Control* (and *wS* at 11:00 and 13:00) (Table S1, Fig. 4b).

For the butterfly *H. hanno,* the ANOVA showed a significant effect of *eT* ($p \leq 0.01$) and interaction between *wS* × *eT* ($p \leq 0.01$) (Table 4). Therefore, the number of total flower visits depended on the temperature in *wS*. Within the same soil water availability condition, the frequency of *H. hanno* visits was greater in *wS* compared to *wSeT*. Within the same temperature condition, *H. hanno* visited the *eT* treatment more frequently compared to *wSeT*. Among all treatments, *H. hanno* visited the *Control* treatment more regularly than *wSeT* (Fig. 4c). In general, the temperature factor contributed to a decrease in *H. hanno* visits to *S. capitata* flowers. For the frequency of visits by *T. angustula* and other bees, the ANOVA showed no significant effects of *wS* and *eT* with no interactions between them (Table S2), indicating that these factors did not affect foraging behavior.

4. Discussion

In a previous study (Alzate-Marin et al., 2021a,b), in which *S. capitata* plants were exposed to warming and elevated atmospheric CO_2 concentrations, we observed positive effects of higher temperature on the number of flowers, earlier flower opening, and enhanced attractiveness for floral visitors, with a direct influence on plant–floral visitor interactions. In the present study, we evaluated under field conditions the effects of +2 °C warming and soil water deficiency on the time of flower opening and closing, sugar concentration in *S. capitata* nectar, and the implications of these changes on interactions with flower visitors. We found alterations in the physiology of *S. capitata* and behavioral activity shifts in detected groups of potential pollinators. Our results confirm our hypotheses and help to clarify the potential effects of future climate scenarios on reproduction, adaptation, and conservation of *S. capitata* (Murcia, 1990; Petanidou and Smets, 1996; Scaven and Rafferty, 2013; Gérard et al., 2020; Alzate-Marin et al., 2021a).

Table 3

Insects collected as visitors during the flowering period from *Stylosanthes capitata* exposed to four treatments: *C* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*. Non-Identified insects (NI).

Order	Family	Tribe/*species*	Treatments				
			C	*wS*	*eT*	*wSeT*	Total
Coleoptera	NI	NI	0	0	1	0	1
Coleoptera	Dasytidae	*Astylus variegatus* (Germar, 1828)	1	1	2	0	4
Diptera	NI	NI	8	9	6	7	30
Lepidoptera	NI	NI	34	42	50	33	159
Lepidoptera	Lycaenidae	*Hemiargus hanno* (Stoll, 1790)	47	65	44	20	176
Hymenoptera	NI	Wasps	7	12	9	5	33
Hymenoptera	Halictidae	Augochlorini					
	Halictidae	*Augochlora* sp. 1	1	0	0	0	1
	Halictidae	*Augochlora* sp. 2	0	0	0	1	1
	Halictidae	*Augochloropsis aurifluens* (Vachal, 1903)	1	1	0	0	2
	Halictidae	*Augochloropsis cupreola* (Cockerell, 1900)	2	0	1	2	5
	Halictidae	*Augochloropsis melanochaeta* Moure, 1950	1	1	1	1	4
	Halictidae	*Augochloropsis* sp. 1	2	2	0	3	7
	Halictidae	*Augochloropsis* sp. 2	0	0	1	0	1
	Halictidae	*Augochloropsis* sp. 3	1	0	0	0	1
	Halictidae	*Temnosoma* sp.	1	0	0	0	1
	Halictidae	*Thectochlora* sp.	0	0	0	1	1
	Halictidae	Halictini					
	Halictidae	*Dialictus* sp.	3	0	3	1	7
	Megachilidae	Anthidiini (NI)	1	0	0	0	1
	Megachilidae	*Ctenanthidium* sp.	0	0	1	0	1
	Megachilidae	*Epanthidium tigrinum* Schrottky, 1905	1	4	2	2	9
	Megachilidae	*Hypanthidium nigritulum* Urban, 1998	0	0	0	1	1
	Megachilidae	Megachilini					
	Megachilidae	*Megachile (Ptilosaroides) neoxanthoptera* Cockerell, 1933	4	1	1	0	6
	Megachilidae	*Megachile (Neochelynia)* sp.	0	0	1	2	3
	Megachilidae	*Megachile* sp. 1	1	1	0	0	2
	Megachilidae	*Megachile* sp. 2	1	2	2	2	7
	Apidae	Apini					
	Apidae	*Apis mellifera* Linnaeus, 1758	271	227	409	279	1186
	Apidae	Exomalopsini					
	Apidae	*Exomalopsis (Exomalopsis) analis* Spinola, 1853	3	4	9	1	17
	Apidae	*Exomalopsis (Exomalopsis) auropilosa* Spinola, 1853	5	5	8	3	21
	Apidae	*Exomalopsis* sp. 2	0	1	3	0	4
	Apidae	Meliponini					
	Apidae	*Paratrigona lineata* Lepeletier, 1836	111	98	98	67	374
	Apidae	*Tetragonistica angustula* Latreille, 1811	23	22	13	14	72
	Apidae	*Nannotrigona testaceicornis* Lepeletier, 1836	0	0	1	0	1
	Apidae	*Trigona spinipes* Fabricius, 1793	2	2	3	2	9
	Apidae	Xilocopini					
	Apidae	*Xylocopa (Neoxylocopa) suspecta* Moure & Camargo, 1988	0	0	0	1	1
Total bees			435	371	557	383	1746
Total floral visitors			967	871	1226	831	2149

The effects of warming on flowering and flower traits depend on the complex interaction of the impacts of temperature on plant physiology and the developmental stages at which plants experience warming, among others (Wahid et al., 2007). The beginning of photosynthesis combined with the action of endogenous auxins (Ke et al., 2018) and warming stimulates early floral opening (Habermann et al., 2019, 2021; Alzate-Marin et al., 2021a). In a previous experiment (Alzate-Marin et al., 2021a), we also observed that warming accelerated flower opening time in *S. capitata* by ~1 h compared to non-warmed treatments. In this study, the flowers of warmed treatments (*eT* and *wSeT*) opened ~45 min earlier than non-warmed plants (*Control* and *wS*). It is likely that the differences in flower opening time in warmed treatments observed in this study (~08:15, 2018 experiment) compared to the previous study (~09:00, 2015 experiment) may be associated with the increase in average diurnal air temperature of ~0.4 degrees between the study periods (Table S3). In the coming years, accelerated *S. capitata* flower opening time could be expected if the temperature continues to increase (Kjøhl et al., 2011; Scaven and Rafferty, 2013), as projected by global climate change scenarios (IPCC, 2021).

Temperature has an impact on the timing of both plant flowering and pollinator activity (Hegland et al., 2009), as observed by Murcia (1990) in *Ipomoea trichocarpa*, with accelerated timing of anthesis on warmer mornings, when its floral visitors arrive. Flowers of plants exposed to elevated temperatures are expected to show significant differences in certain characteristics that could affect how available, attractive, and profitable their rewards are for insect visitors and pollinators (Wahid et al., 2007; Scaven and Rafferty, 2013). Here, in the early hours of the day, *A. mellifera* bees visited the warmed treatments more frequently, regardless of whether the plots were irrigated or not. Meanwhile, *P. lineata* showed a preference for the *Control* treatment between 11:00 and 13:00. Around midday, as the air temperature increased, we also observed significant adverse interaction (*A. mellifera*) and additive effects (*P. lineata*) of soil water deficit (*wS*) combined with warming (*eT*) on the frequency of insect visits. The decrease in the number of insect flower visitors recorded around noon in warmed plots may be associated with flower closure observed at this time.

Climatic conditions can also modify the supply of resources such as pollen, sugar, and nectar since plants have different mechanisms to deal with warming and water stress (Takkis et al., 2015; Petanidou and Smets, 1996; Phillips et al., 2018; Descamps et al., 2018, 2021). In a study on *Silphium perfoliatum* L. (Asteraceae), in which the potential energy plant native to temperate North America was planted in northern Germany, the mean nectar sugar production and honeybee visitation were approximately three and two times greater, respectively, in irrigated plots compared to rainfed plots (Mueller et al., 2020). Herein, under irrigated and non-irrigated conditions, besides the stimulation of early *S. capitata* flower

Fig. 3. Bees (a) *Paratrigona lineata*, (b) *Augochloropsis cupreola*, (c) *Exomalopsis auropilosa*, (d) *Megachile* (*Neochelynia*) sp., (e) *P. lineata* with pollen grains in the ventral region (white arrow), and (f) the butterfly *Hemiargus hanno*, collected while foraging on *Stylosanthes capitata* flowers in the 2018 experiment. Photos a to e were taken with a Leica S APO stereomicroscope at 12.5 × magnification, except for photo d which was photographed at 10 ×. Photo f was taken with a Zeiss Discovery.V12 stereomicroscope at 8 × magnification. Scale bar = 1 mm.

opening, and more frequent visits of *A. mellifera* in the early morning, the 2 °C warming also increased sugar concentration in the nectar.

Because of the minimal volume, it was not possible to measure with precision the total amount of nectar in *S. capitata* flowers. Nevertheless, we observed different nectar viscosity between treatments (Fig. S2). Visually, we found a greater quantity of nectar in the flowers of the *Control* than in the other treatments, especially in comparison to the *wSeT* treatment. The changes in floral characteristics due to the effects of warming and water stress may result in significant alterations in plant–pollinator relationships (Memmott et al., 2007; Hegland et al., 2009; Hoover et al., 2012; Scaven and Rafferty, 2013; Takkis et al., 2018; Mueller et al., 2020; Alzate-Marin et al., 2021a,b).

Floral temperature is a signal that floral visitors and pollinators use to identify flowers. Bees, for example, prefer to collect warm nectar from flowers at low ambient temperatures (Dyer et al., 2006) and can detect differences in overall flower temperature using thermal sensors in their antennae and tarsi (Hammer et al., 2009). Flower temperature patterns can help bees during foraging, as observed in several species by Harrap et al. (2017). In this study, we observed that floral stimuli resulting from warming increased *A. mellifera* visitation in the early morning. However, water stress on its own and interacting with elevated temperature reduced *A. mellifera* visits in the early morning and during the day's hottest hours (~noon), respectively. On the other hand, *P. lineata* showed a preference for non-warmed treatments after 11:00, with adverse additive effects between water stress and warming. Using a thermal infrared camera, we observed that the mean temperatures of *S. capitata* flowers at about 12:00 in the warmed treatments (*eT* and *wSeT*) were 4.6 and 2.1 °C higher, respectively, compared to the *Control*. Interestingly, the temperature of non-warmed *wS* flowers was 2.9 °C higher than the *Control* (Fig. 1b), which may be related to a similarly observed effect in leaves due to a reduction of stomatal conductance and transpiration as a result of water stress (Habermann et al., 2021). The observed decrease in floral visitors around 12:00 in warmed treatments is likely related to reduced floral resources due to earlier flower closing combined with shorter flower lifetime rather than floral temperature. These results suggest a mismatch in plant–flower visitor interactions as a result of the effects of warming (Gérard et al., 2020). In previous studies, we observed a higher photosynthetic rate (Habermann et al., 2019), greater partitioning of biomass to leaves (Martinez et al., 2014), and a larger number of flowers and inflorescences (Alzate-Marin et al., 2021a; Habermann et al., 2021) in well-irrigated *S. capitata* plots exposed to

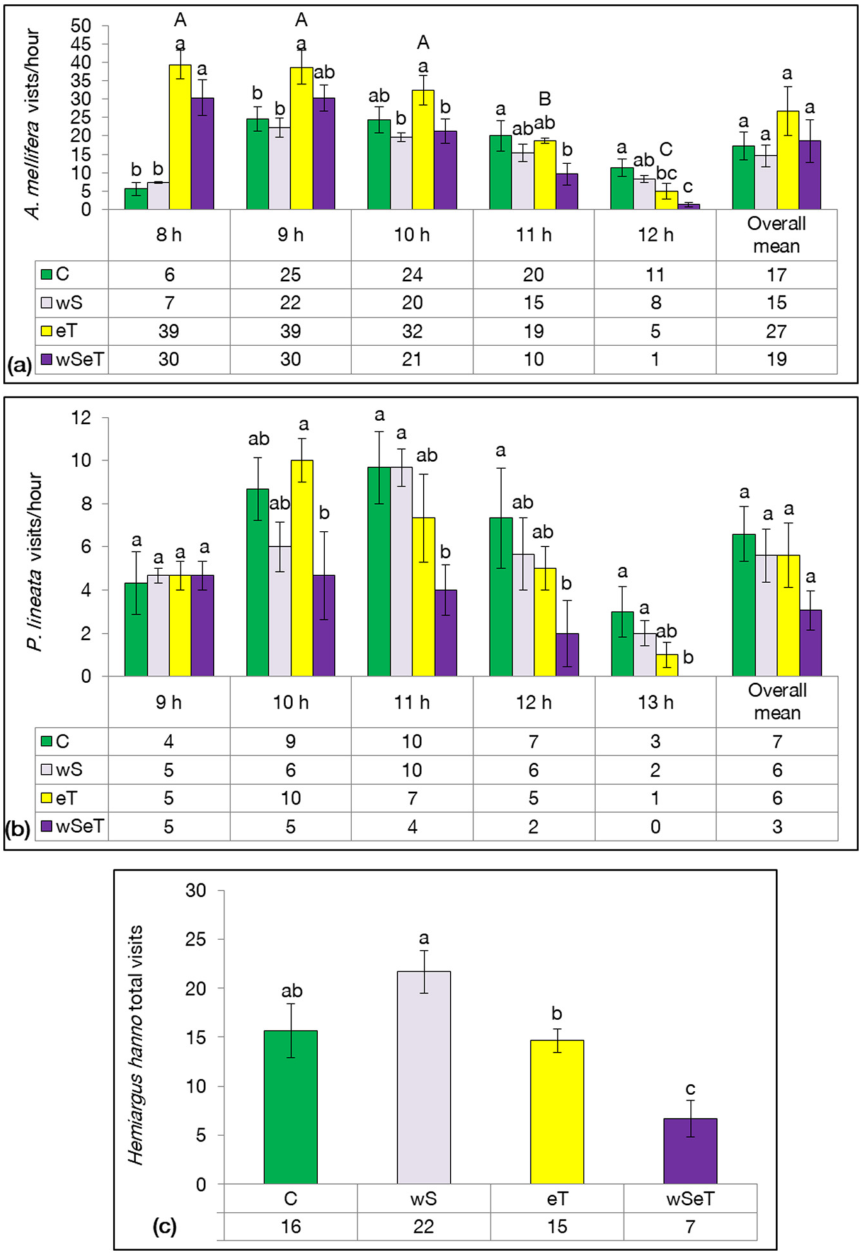

Fig. 4. Mean number of visits to *Stylosanthes capitata* flowers by bees (a) *Apis mellifera* and (b) *Paratrigona lineata* per hour, and (c) total visits for the butterfly *Hemiargus hanno* as a function of treatments (*Control* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*). In a and b, lowercase letters compare the effects of treatments in each hour. In a, capital letters compare the effects of hours in the *eT* treatment. Treatments followed by the same letter do not differ statistically based on the LSD Fisher test at 5%.

warming when compared to *Control* plots. Here, we observed significant effects of warming on early flower opening, with more frequent flower visits and increased floral resources with greater sugar concentration in the nectar. However, water stress can induce lower rates of photosynthesis and reduce stomatal conductance (g_s) and leaf dry mass accumulation (Habermann et al., 2021), which could explain the detrimental effects of these stresses on the availability of floral resources in warmed and water stress conditions (Takkis et al., 2018), especially in the hottest hours of the day.

In this study, we considered the Africanized honeybee *A. mellifera*, and the bees of the tribes Augochlorini, Exomalopsis, and Megachilidae, as potential *S. capitata* pollinators as they triggered the wing-keel complex and promoted the transfer of pollen between flowers. Furthermore, *P. lineata* was identified as a potential pollinator not only through sternotrobic pollination, but also considering the pollen found on the corbiculae from accessing the flower, which is likely facilitated by *A. mellifera* or other bees, as was also observed by Carvalho et al. (1999). This is a significant behavior change when compared with our study in 2015 (Alzate-Marin et al., 2021a). For the butterfly *H. hanno*, *S. capitata* appears to be a source of nectar. Since we only analyzed a few individuals and could not verify oviposition in the flowers, its function as a potential pollinator or host must be further examined.

The floral visitors identified here occur naturally in the neighboring environment around the experiment and were not exposed to the treatments; however, they may already be experiencing the effects of current climate change such as heatwaves and drought (IPCC, 2021). Even if minimal, the predicted increased temperatures are likely to have more harmful consequences for tropical insects with a narrow range of temperatures suitable for survival than insects from higher latitudes (Deutsch et al., 2008; Kjøhl et al., 2011).

Table 4
Analysis of variance for mean flower visiting frequency by the bees *Apis mellifera* and *Paratrigona lineata*, and the butterfly *Hemiargus hanno* for *Stylosanthes capitata* exposed to the following treatments: *Control* - irrigation and ambient *Tcanopy*; *wS* - non-irrigation and ambient *Tcanopy*; *eT* - irrigation and elevated *Tcanopy* [+2 °C above ambient *Tcanopy*]; and *wSeT* - non-irrigation and elevated *Tcanopy*.

Source of variation	*A. mellifera*		*P. lineata*[&]		*H. hanno*	
	Visitors/hour ± SE	*P-value*	Visitors/hour ± SE	*P-value*	All visits ± SE	*P-value*
Control[a]	17.2 ± 3.8	–	6.6 ± 1.3	–	15.7 ± 2.7	–
wS	14.6 ± 3.0	0.000**	5.6 ± 1.2	0.003**	21.7 ± 2.2	0.64 ns
eT	26.8 ± 7.0	0.000**	5.6 ± 1.5	0.002**	14.7 ± 1.2	0.01*
wS × *eT*	18.6 ± 5.8	0.041*	3.1 ± 0.9	0.117 ns	6.7 ± 1.9	0.01*
Hours	19.3 ± 2.6	0.000**	5.2 ± 1.1	0.000**	–	–
Hour × *wS*	–	0.785 ns	–	0.284 ns	–	–
Hour × *eT*	–	0.000**	–	0.121 ns	–	–
Hour × *wS* × *eT*	–	0.828 ns	–	0.914 ns	–	–

*Significant at 5% probability, **Significant at 1% probability, ns = Not significant. & = Transformed data (Box-Cox software PAST).

[a] The mean and standard error (SE) for *Control* is presented for reference as *Control* is not a source of variation in the analysis of variance.

5. Conclusions

Global warming is a significant threat to the relationships and mutualisms between pollinators and their host plants. The field-realistic experiments conducted in the T-FACE facility enabled us to assess the effects of warming and water stress on shifts in plant–flower visitor interactions for the tropical forage legume *S. capitata* and foraging behaviors of visitors. We demonstrated a significant effect of 2 °C warming on floral biology, flower resource production, and a mismatch of plant–floral visitor relationships, especially around midday when combined with water stress. In summary, these results provide a better understanding of the effects of climate change on flower visitor interactions with *S. capitata*, and suggest that warming and soil water deficits could affect the reproductive success of this species in tropical belts.

Statement of human and animal rights

This article does not contain studies with human participants or vertebrate animals.

CRediT authorship contribution statement

Raquel Pérez Maluf: Investigation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Ana Lilia Alzate-Marin:** Conceptualization, Supervision, Resources, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Carolina Costa Silva:** Investigation, Formal analysis. **Fernando Bonifácio-Anacleto:** Formal analysis, Resources. **Ivan Schuster:** Formal analysis, Writing – review & editing. **Renato de Mello Prado:** Resources, Writing – review & editing. **Carlos A. Martinez:** Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Investigation, Resources, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

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