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Research Article

Flower display and temperature in Royal irises (*Iris* section *Oncocyclus*, Iridaceae)

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Abstract

Large flowers are typically more conspicuous to pollinators and are associated with more nutritional rewards than small flowers. Flower size can also determine flower temperature, which can be a reward for flower-visiting insects. Nectarless Royal irises offer overnight shelter and morning warmth to male *Eucera* bee pollinators. A dark spot on their lower tepals (black patch) may act as a visual cue and contribute to flower heating. Here, we examined the association of floral display (i.e. flower size and black patch size) with flower temperature and female fitness (i.e. seed set) in Royal irises populations across an aridity gradient. First, we tested pollinator preference using artificial flowers of varying sizes. Next, we assessed associations between flower and black patch size, flower warming rate and female fitness. Finally, we manipulated flowers in the field to determine if the black patch influences heating. Pollinators preferred larger artificial flowers for overnight shelter. However, selection for larger flowers was found only in the population with the smallest flowers. No association was found between flower/black patch size and floral heating in natural populations, and the black patch did not affect flower heating. Flowers reached the temperature threshold for bees to start flying (18 °C) 10–35 min faster than ambient air. We conclude that the large flower size in the Royal irises serves as a visual signal, advertising for night shelter and flowers—independently of the size—heat up faster in the morning than ambient air. Flowers thus, potentially offer a 'head start' to the flower-dwelling bees, allowing them to warm up more quickly than in outside conditions.

Keywords: Royal irises, *Oncocyclus*, pollination, selection, floral traits, shelter reward

皇家鸢尾的花部展示性状与花温特征

摘要：大型花通常对传粉者更具吸引力，并具有更高的营养回报。同时，花的大小也会影响其温度，进而作为昆虫访花的潜在回报。无蜜皇家鸢尾为雄性长距蜂(*Eucera*属)提供夜间庇护与晨间取暖场所，其下

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花被片上的深色斑块(黑斑)可作为视觉信号，并有助于花部加热。本研究在干旱梯度上，探究了皇家鸢尾花部展示性状(花径与黑斑大小)与花温及雌性适合度(种子结实率)之间的关系。研究首先利用不同大小的人造花测试传粉者偏好，随后分析自然种群中花与黑斑大小、花部升温速率及结实率之间的关联，最后在野外通过控制实验验证黑斑是否影响花部加热。结果表明，传粉者更偏好较大的人工花作为夜间庇护所。然而，仅在自然花朵最小的种群中观察到对大花的选择性压力。在自然种群中，花或黑斑的大小与升温能力之间未发现显著关系，黑斑对花部加热也无显著影响。皇家鸢尾花部在早晨可较环境温度更早达到蜜蜂起飞所需的温度阈值(18 °C)，提前时间为10–35分钟。综上所述，皇家鸢尾的大型花作为视觉信号吸引传粉者夜宿，而无论花部大小，其均能在清晨快速升温，为栖居其中的蜜蜂提供“热启动”优势，较外部环境更快恢复活动。

关键词：皇家鸢尾，鸢尾(*Oncocyclus*)，传粉，选择作用，花部性状，庇护回报

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INTRODUCTION

Plants exhibit an immense diversity in flower color, shape and size, which attracts pollinators who transport pollen from one flower to another, ensuring their reproduction (Faegri and van der Pijl 1979; Galen 1997; van der Kooi *et al.* 2021; Willmer 2011). In exchange, pollinators are typically rewarded with food, such as pollen and/or nectar (Baker and Baker 1979; Nicholls and Hempel de Ibarra 2017; Parachnowitsch *et al.* 2019; Roulston and Cane 2000). Although there are also other types of rewards, such as resins (Armbruster *et al.* 2005; Chui *et al.* 2021; Pélabon *et al.* 2012), shelter (Dafni *et al.* 1981; Gaglianone 2000; Hemborg and Bond 2005) or heat (Herrera 1995; Kevan 1975; Sapir *et al.* 2006; Seymour *et al.* 2003).

The size of a flower can serve as a visual indicator of the presence of rewards such as nectar or pollen. It was previously shown that large flowers can have higher energetic and nutritive rewards than smaller ones (Armbruster *et al.* 2005; Ortiz *et al.* 2021; Stanton and Preston 1988; van der Kooi *et al.* 2023), and therefore pollinators can use flower size to estimate the amount of reward. In addition, large flowers are easy to detect from a distance (Spaethe *et al.* 2001), which may result in pollinator-mediated selection for larger flowers (e.g. Campbell 1996; Galen 1989; Johnston 1991; Lavi and Sapir 2015; Lebel *et al.* 2018; Tavares *et al.* 2016). By contrast, environmental conditions, such as water availability, may counter the selection favoring smaller flowers (Galen *et al.* 1999; Hu *et al.* 1998; Kuppler and Kotowska 2021; Rose-Person *et al.* 2024; Zhang and Brodribb 2017).

A lot is known about the influence of biotic (e.g. pollinators) and abiotic factors (e.g. water availability) on flower size and its relationship with flower rewards such as nectar and pollen (e.g. Gallagher and Campbell 2017; García *et al.* 2023). However, for other reward types, such as heat, plant-pollinator dynamics are much less clear (reviewed by van der Kooi *et al.* 2019). Flowers can obtain heat from flowers that are thermogenic (Seymour and Matthews 2006; Seymour and Schultze-Motel 1997) or that capture solar radiation (Rejšková *et al.* 2010; Dietrich and Körner 2014; van der Kooi *et al.* 2019). Heat accumulation inside flowers constitutes a reward for insect pollinators, especially when ambient temperatures are low, for example in the early morning and in Arctic or Alpine regions (e.g. Kevan 1975). When the ambient temperature is low, elevated flower temperatures can serve as a reward for pollinators and are beneficial for pollen and seed development (van der Kooi *et al.* 2019).

Royal irises (*Iris* section *Oncocyclus*) are an ideal group to study flower size in relation to flower temperature because their relatively large flowers and high phenotypic variation within and among populations and species (Roguz *et al.* 2020; Sapir *et al.* 2002). Royal irises are a monophyletic group that comprises around 30 species, with flowers that range in size from 1.25 cm diameter in *Iris sprengeri* to 16.5 cm diameter in *Iris gatesii* (Roguz *et al.* 2020). Unlike their close relatives, these flowers do not produce any nectar (Mathew 1989). Royal irises are pollinated primarily by male solitary *Eucera* bees that use flowers as overnight shelters, transporting pollen among flowers as they look for shelter at sunset (Monty *et al.* 2006; Sapir *et al.* 2005; Vereecken *et al.* 2013; Watts *et al.* 2013). These irises produce one self-incompatible flower per stem, with a characteristic dark spot (hereafter referred to as the black patch) located on the lower tepals at the entrance of the shelter (Avishai and Zohary 1980; Monty *et al.* 2006; Sapir *et al.* 2005; Watts *et al.* 2013), which might guide pollinators to the reward, functioning similar to nectar guides. At sunrise, the temperature within flowers increases faster than ambient temperatures. Floral heating in the morning sun allows flower-dwelling bees to emerge earlier than those that overnight on the bare ground or cervices (Sapir *et al.* 2006). In addition, the black patch at the entrance of the flower tunnel may contribute to floral heating and be a cue to pollinators. Thus, we hypothesize that the black patch functions not only as a visual guide for pollinators but also plays a role in flower warming. Royal irises in Israel and Palestine are naturally occurring along a north–south aridity gradient, showing a decrease in flower size toward the southern desert region, with intra- and interspecific variation in flower size and black patch size (Sapir *et al.* 2002). A recent study has shown that larger flowers have higher fruit and seed set in natural populations, suggesting positive selection for larger flowers (Lozada-Gobilard *et al.* 2023). Indeed, selection for larger flowers by pollinators was previously reported (Lavi and Sapir 2015), but flower size highly depended on water availability over the years in arid habitats (Lozada-Gobilard *et al.* 2022). Interestingly, in those habitats, water availability did not affect the size of the black patch over the years, suggesting that the size of the black patch is an important trait, probably signaling the entrance to the shelter (Lozada-Gobilard *et al.* 2022).

Flower size and black patch size may act as an honest indicator for the size of the flower tunnel (i.e. shelter reward); however, it is not clear how these traits relate to the flower tunnel temperature (i.e. heat reward) (Lozada-Gobilard *et al.* 2023). In addition, the black patch size may also contribute to the heating of the flower. However, how the increase in floral temperature relates to flower size and black patch size, whether the black patch plays a role in floral heating, and whether flower size can serve as a cue for pollinators to estimate the putative heat reward, remains unknown.

In the present study, we investigated the association between flower size, black patch size and flower temperature in Royal irises. The unique pollination system, alongside their large flower size and the black patch of these irises, has motivated three different but complementary experiments on floral display and reward. A previous study suggested that pollinators select for larger flowers based on maternal fitness measurements (Lavi and Sapir 2015), but in this study, pollinators were not observed directly; their choice for larger flowers was inferred from a selection experiment. To explore directly the effect of flower size on the attraction of the pollinators, we performed an experiment with artificial iris flowers that vary only in size (Experiment 1). The minimum ambient temperature at which *Eucera* bees begin to fly and are able to forage is ~18 °C (Stone and Willmer 1989). Therefore, understanding how quickly flowers warm up to this temperature is crucial for the bees' take-off and foraging. We tested whether the time needed to warm up to 18 °C varied between populations and whether time to warm up is associated with flower size and black patch size (reward; Experiment 2). In addition, we recorded seed set in these individuals to tested for selection on flower size and black patch size within populations (selection; Experiment 2). Finally, to determine whether the time needed to warm up to 18 °C is influenced by floral morphology, we manipulated the "flower tunnel" (i.e. shelter) and black patch (Experiment 3).

MATERIALS AND METHODS

Study species

In this study, we studied two *Iris* species belonging to the *Oncocyclus* section: *Iris atropurpurea* Baker and *Iris petrana* Dinsm. whose natural populations occur along the aridity gradient in Israel (Fig. 1a). Flowers of *I. atropurpurea* are uniformly colored dark purple, while flowers of *I. petrana* exhibit a wide range of colors from dark red, almost black to light purple and pink. Like all Royal irises, these species have a characteristic dark spot, the black patch, in the middle of the low tepals (Fig. 1b–d). Both species flower for 2–3 months, with *I. atropurpurea* flowering earlier, starting at the end of January until mid-March, and *I. petrana* flowering from March to April. Flowers produce fruits at the end of the flowering season, and seeds mature in 2–3 weeks. Both species can reproduce vegetatively via rhizomes, producing many individual leaf fans (hereafter ramets) and flowering stems in well-defined patches that are easy to distinguish. A "genet'' (sensu Harper 1977) or "genotype" is a genetically identical individual that developed from one seed and, via vegetative growth, has produced one or more ramets (Scrosati 2002). We assume that ramets occurring in these defined patches belong to the same individual or genotype.

Experiment 1: Pollinator visitation in relation to flower size

Artificial flowers

To test how pollinators respond to size variation, we used artificial flowers. Different studies showed the usefulness of artificial flowers for manipulating specific floral traits, such as corolla

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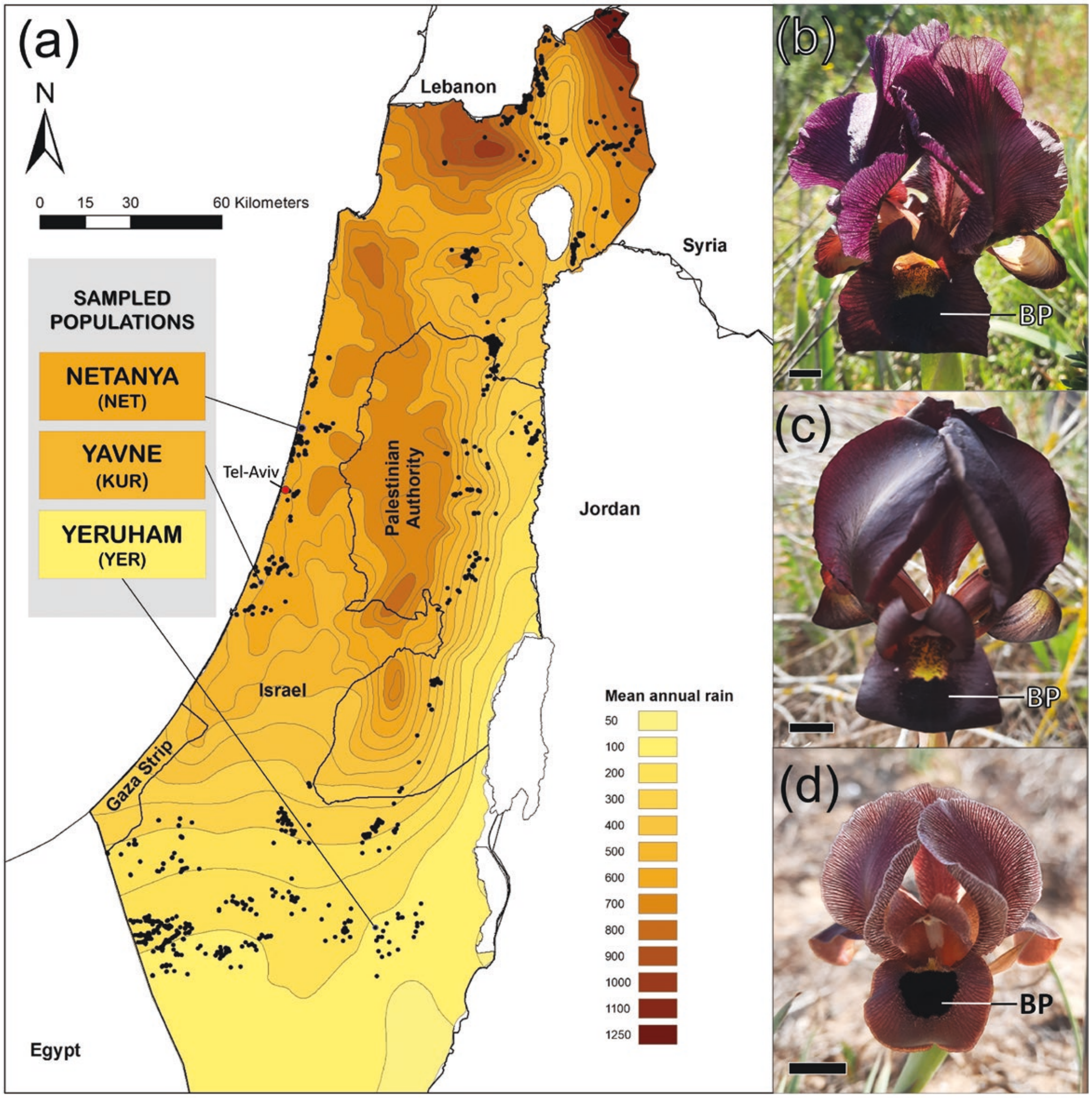

Figure 1: Study sites and species. (a) Map shows the natural populations of Royal irises (*Oncocyclus*) along an aridity gradient mainly determined by mean annual rain in Israel. We sampled three populations: Netanya (NET), Yavne (KUR) and Yeruham (YER). Mean annual rain: 600 (NET), 500 (KUR) and 200 mm (YER). First two populations correspond to *Iris atropurpurea* and the last one to *Iris petrana*. Black points denote the occurrence of wild *Iris* populations (database courtesy of Y. Sapir). The right panel shows examples of measured flowers in (b) Netanya, (c) Yavne and (d) Yeruham. Tel Aviv city (red dot) shows the location of the Botanical Garden where the artificial experiment was performed. The characteristic "black patch" located in the lower petals is shown as BP. Scale bar: 1 cm. Pictures: S. Lozada-Gobilard.

shape or color (e.g. Campos *et al.* 2015; Fenster *et al.* 2006; Policha *et al.* 2016). We created a system of artificial flowers, designed based on the shape and size of *I. atropurpurea*. We built 120 models in 6 size categories (Supplementary Table S1) using a combination of computer-designed 3D technology and hand-crafted flowers of hardened black satin cloth (Supplementary Method S1 and Fig. S1). Size categories increased progressively by 25% of the original size, beginning with the smallest possible tunnel entrance that could be crafted and where a male *Eucera* bee could enter easily (tested with pinned insects). We multiplied the length and width of the artificial flowers to calculate the flower projection size—that is the scale of the visual signal that a bee would observe (Supplementary Table S1). Size categories 2–5 of the flower models correspond to the natural size range of *I. atropurpurea* (Sapir *et al.* 2002). The artificial flowers were all in the same color and shape, varying only in size, providing a non-biological system to test the isolated effect of flower size on pollinator behavior.

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Pollinator preference—Artificial flowers

To test the hypothesis that pollinators prefer large flowers, the artificial flowers were placed in a randomized array in a semi-natural garden at Tel Aviv University Botanical Garden (TAUBG; 32°06′ N, 34°48′ E). Local vegetation represents a Mediterranean habitat, supporting a large population of *Eucera* bees and other wild bee species (Y. Sapir, personal observation). There were no irises or other flowering species in the open collection where we set up the experiment, thus minimizing the potential for bee learning to influence behavior. Flowers were mounted on wooden skewers approximately 40 cm above the ground and 50 cm apart from each other (Supplementary Fig. S1). Between 1st and 16th March 2016, we recorded all bees that sheltered within the tunnels of the artificial flowers in the evening. Flowers were checked within ±30 min of sunset, when all bees would have settled inside shelters for the night (Sapir *et al.* 2006). The average temperature in Tel Aviv in March is 16 °C and the precipitation is 44 mm.

We tested the effect of flower size category on the total number of bees observed each night in each model flower using a generalized mixed model with a Poisson family distribution, including day as a random effect. Pairwise Tukey comparisons were calculated with the function "*glht*" from the R package "*multcomp*".

Experiment 2: Flower and black patch sizes and its relationship with flower temperature and fitness

Natural populations

The study in natural populations was conducted in two populations of *I. atropurpurea* and one of *Iris petrana*. One population of *I. atropurpurea* is in Netanya Iris reserve (NET; 32°170′ N, 34°500′ E, 37 m a.s.l.), 26 km north of Tel Aviv, and the other near Yavne (KUR; 31°53′25.4″ N, 34°42′35.60″ E, 15 m a.s.l.), 15 km south of Tel Aviv. The *I. petrana* population is in the Yeruham Iris Nature Reserve south of Israel (YER; 31°01′14.46″ N, 34°58′21.4″ E, 549 m a.s.l.). These three populations are distributed along a latitudinal gradient from north to south, with 600, 500 and 200 mm of mean annual precipitation, respectively (Fig. 1a). The average temperature from February to April in Netanya is 18–23 °C, 19–25 °C in Yavne and 18–26 °C in Yeruham.

Flower and black patch size measurements

Flower size was measured in the field using calipers during the flowering season (January–April) of 2021. We measured all open, viable flowers per genotype; wilted flowers were not measured but counted to estimate the total number of flowers per genotype. We calculated flower size as a projection size in cm^2 by multiplying the length by the width of the flowers (see figure 1 in Lozada-Gobilard *et al.* 2023). In addition, we photographed flowers with a scale in the field and calculated frontal and black patch area using ImageJ software following a standardized protocol (Schneider *et al.* 2012). The frontal area corresponds to the whole flower frontal view, where one of the three black patches is centered. The relative area of the black patch was calculated in relation to the frontal area (Supplementary Fig. S2). To test whether flower size varies among populations, we fitted a linear model including the average flower size per individual (genotype) as the response variable and populations as the predictor. Similar methodology was implemented for the black patch area and relative area. Additionally, to test whether flower size is positively correlated to black patch area within populations, we fitted a linear model including population as a covariate. Data were log-transformed to improve normality for the significance test. Pairwise comparisons between populations were performed with a post-hoc Tukey test from the R package "*stats*". An overview of sample sizes and mean values per flower and genotype can be found in Supplementary Table S2.

Flower temperature measurements

Flower temperature in natural populations (NET = 45 flowers, 14 genotypes; KUR = 34 flowers, 12 genotypes; YER = 25 flowers, 20 genotypes) was measured using thermocouples (DLogMate-2TCK), with an accuracy of ±0.5 °C. To test whether large flowers warm up faster than small flowers after sunrise, we measured flower temperature (°C) at intervals of 5–10 min, starting a few minutes before sunrise until approximately 2.5 h after sunrise. Temperature (in °C) was obtained from the air cavity inside the pollination tunnel, in the tunnels facing east (sunrise direction); and of the ambient air, outside the pollination tunnel right next to the flower also facing east (Sapir *et al.* 2006).

We were interested in how flowers warm with the morning sunlight, so we considered flowers measured under sunny conditions only, obtaining a total of 71 flowers and 33 genotypes (NET = 33 flowers, 10 genotypes; KUR = 19 flowers, 8 genotypes; YER = 19 flowers, 15 genotypes) for the analysis. For each flower, we obtained absolute temperatures of the air inside the flower tunnel and outside ambient air. To estimate

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the rate of temperature change, we fitted a linear regression based on these absolute temperatures from sunrise up to 140–150 min after (individual fits per flower can be seen in Supplementary Figs S3–S5). To test whether temperature increase slopes varied among populations and between inside and outside the flower, we performed a linear model including population and air temperature position (inside *vs*. outside flower) as predictors. Slopes were considered as response variables and log-transformed to obtain normality.

To determine whether the temperature inside the flower tunnel increases faster than the ambient temperature, we calculated the time (in minutes after sunrise) required for the flowers to reach 18 °C, because at this temperature, *Eucera* bees begin to fly and forage (Stone and Willmer 1989). We calculated linear approximations to 18 °C per flower using the previously fitted model parameters. Only a few flowers from the YER population were considered, since most of them did not achieve 18 °C (Supplementary Fig. S5). To test whether there is a difference in temperature between inside and outside the flower, we performed a paired *t*-test per population using the R package "*stats*".

Additionally, we calculated the difference between Ambient Air–Tunnel flower, where positive values indicate that the flower tunnel warms up faster than the ambient air, while negative values indicate the opposite. We averaged the time difference across flowers in each genotype and tested its association with mean flower and black patch sizes using a generalized linear model including population as a predictor. Time difference, flower size and black patch sizes were log-transformed to achieve normality.

Fitness and selection analyses

Around 2–3 weeks after the end of the flowering in each population, we counted the number of fruits in each genotype and collected up to three mature fruits that were brought to the laboratory and dried at air temperature until full maturation. As a measure of maternal fitness, we calculated fruit set and seed set. Fruit set (%) was calculated as the number of fruits per genotype, divided by the number of flowers per genotype. To calculate seed set, we calculated the mean number of seeds per fruit and the total number of seeds per genotype (mean number of seeds per fruit × total fruits per genotype).

Unfortunately, in the winter of 2020–2021, rain was very delayed, extremely affecting the growth of *I. petrana* in Yeruham, and as a result, only a few individuals flowered. Few individuals were measured for size and temperature, and none of them produced fruits (YER = 33 flowers, 28 genotypes). In 2017, the same population was monitored, and flower size, black patch size, number of fruits and viable seeds were recorded (Supplementary Table S2). We did not find a significant difference in flower size, mean annual rain and mean annual temperature between 2017 and 2021 (Supplementary Fig. S6), so we used data only from 2017 for selection analysis in Yeruham. Hereafter, unless stated otherwise, data presented and analyzed correspond to 2020/2021 season. We used a linear model to compare the mean number of seeds per fruit among the three populations (NET = 57; KUR = 47; YER = 171 flowers); pairwise comparisons between populations were performed with a post-hoc Tukey test using the R package "*stats*". Significance was inferred after square-root transformation of the mean number of seeds per fruit to achieve normality.

To estimate the extent and direction of natural selection on flower size, we used regression analyses, following the method from Lande and Arnold (1983). For each population separately, we calculated relative fitness (number of seeds/mean of the population) and standardized flower and black patch sizes ((individual size—population mean)/population SD), and then these values were averaged per genotype (at plant level) to be used for the selection analyses. To test whether there is directional selection on flower and black patch size, we implemented a linear model per population separately, including size traits (i.e. flower, black patch) and number of flowers per genotype as a covariate, as a proxy for plant size. Significance was inferred after square-root transformation of relative fitness to achieve normality.

Experiment 3: Flower warming mechanism, the role of the black patch

Black patch anatomy

The pollination tunnel (i.e. shelter) is formed by the petaloid style with a stigmatic surface at the tip, one anther is inside and below, the lower tepal has hairs and the black patch is located at the center outside the shelter (Figs 1 and 5a). We obtained cross-sections from flowers of three individuals of *I. atropurpurea* from the NET population. We measured the petal's thickness at the base, in the middle and at the tip using a caliper (van der Kooi and Stavenga 2019). Given the large differences in thickness and visual appearance of a single petal, we further investigated the interior and surface anatomy of the tepals. Flowers of *I. atropurpurea* from the NET population ($n = 9$) were cut

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into small pieces, fixed in FAA50 [3.7% formaldehyde, 50% ethanol and 5% acetic acid] and dehydrated in an ethanol series [50%, 70%, 90%, 95% and 100% (v/v)]. Samples were embedded in Technovit 7100 resin (Kulzer, Germany) with the addition of 0.6 mL polyethylene glycol 400. Samples were cut into 5 µm thick sections with solid tungsten carbide blades (Sollex AB, Malmö, Sweden) on a rotary microtome (Beck, London), stained with toluidine blue (0.05%, aqueous) and photographed with use of an inverted Nikon Diaphot 300 microscope (Nikon, Amsterdam, Netherlands), coupled with a Nikon D3200 digital camera (Nikon, Amsterdam, Netherlands) following Kraaij and van der Kooi (2020).

Black patch manipulations

To test whether the black patch contributes to the heating of the tunnel, we set up three manipulations: (i) "No ceiling", where we removed the cover of the tunnel, the petaloid style together with the stamens ($n = 9$); (ii) "Only patch", where we removed all the area surrounding the black patch ($n = 10$); (iii) "Covered patch" where we covered the area of the black patch with a cut piece of another low tepal ($n = 9$). All manipulations were set before sunrise and the temperature in the manipulated flowers was compared with control flowers ($n = 40$) (see Fig. 5). We calculated and compared the flower's warming rate (measured in time that a flower needs to warm up to 18 °C, see above) between different manipulations of the black patch in a subset ($n = 68$) of flowers in *I. atropurpurea* populations NET (50 flowers, 8 genotypes) and KUR (18 flowers, 5 genotypes). Due to the very low availability of flowers in YER (*I. petrana*), we did not perform this experiment in this population. Only measurements under sunny conditions were considered. Due to the very low number of flowers per manipulation, we pooled together both datasets and applied a linear mixed model including treatment as a predictor and population as a random effect. Times were square-root transformed to achieve normality. Pairwise Tukey comparisons were calculated with the function "*glht*" from the R package "*multcomp*".

RESULTS

Experiment 1: Pollinator visitation in relation with flower size

To test the effect of flower size on pollinators' visitation, we monitored the total number of bees observed each night in each flower of the six size categories. Over the course of 16 evenings, we observed a total of 101 bees in the tunnels of artificial flower models (Fig. 2a). Bees sheltered in intermediate and large flowers (sizes 4 and 5) significantly more often than either the very large (size 6) or small flowers (sizes 1–3) (Fig. 2b; GLMM (Poisson) $\chi^2_5 = 13.69$, $P < 0.05$).

Experiment 2: Flower size and its relationship with flower temperature and fitness

Flower and black patch sizes

There was a significant difference in flower size, calculated as projection size (length × width), between populations in association with the latitudinal aridity gradient (Fig. 3). Flowers in the northern site NET (Netanya) were the largest (Mean ± SE; 48.9 ± 0.8 cm^2), followed by the flowers in the semi-arid site KUR (Yavne, middle) (37.2 ± 0.5 cm^2) and flowers in the desert site YER (Yeruham, south) were the smallest (18.6 ± 1.0 cm^2) (Fig. 3a; Supplementary Fig. S7a–d; LM, $F_{2,115} = 119.01$, $P < 0.001$). Total area of the black patch was different between populations (LM, $F_{2,260} = 71.21$, $P < 0.001$; Fig. 3b; Supplementary Fig. S7e), due to a significant difference between YER and the two *I. atropurpurea* populations, which did not significantly differ NET: 1.65 ± 0.7 cm^2; KUR: 1.57 ± 0.6 cm^2, while the YER (*I. petrana*) population was 38% smaller (0.62 ± 0.3 cm^2). Relative area of the black patch also varied among populations (LM, $F_{2,254} = 22.29$, $P < 0.001$; Supplementary Fig. S7f), the NET population was 20% smaller than KUR, but no different from YER. Within populations, flower size was positively correlated to black patch area in NET and YER populations ($P < 0.001$, Supplementary Fig. S8a).

Flower temperature

In all three populations, absolute temperature increased over time, up to 140–150 min after sunrise, both within flower tunnel and ambient air outside the flower (Fig. 3c). Slopes calculated from absolute temperatures per genotype differed among populations (LM, $F_{2,136} = 145.8$, $P < 0.001$) and between inside and outside the flower (LM, $F_{2,136} = 15.5$, $P < 0.001$). Slopes were higher inside the pollination tunnels, compared to ambient air in all three populations but this difference was significant in YER population only (Tukey test, $P < 0.05$, Supplementary Fig. S9). The required time for a flower to warm up to 18 °C differed among populations (~110–130 min; LM $F_{2,46} = 6.3$, $P < 0.01$), but it was significantly different compared to ambient air in KUR ($P < 0.05$) and YER ($P < 0.01$) (Fig. 3d). This time difference (Air–Tunnel) at

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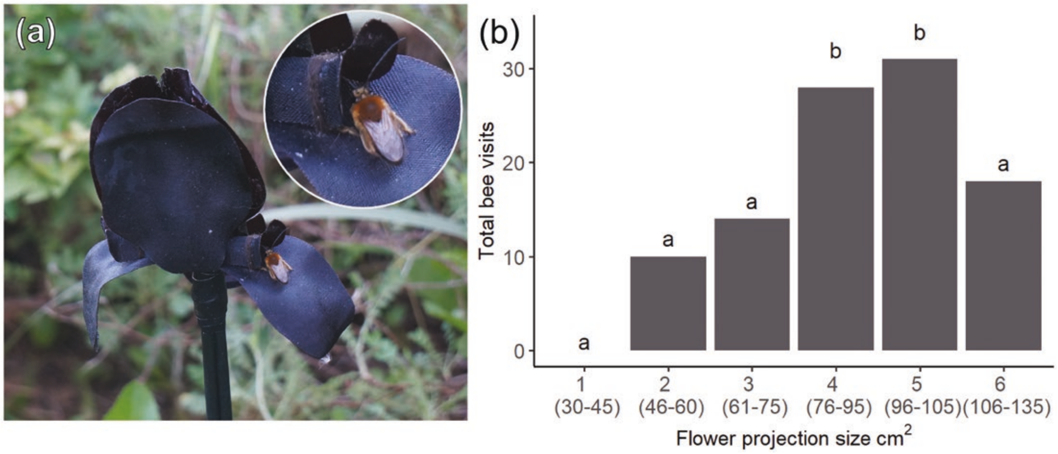

Figure 2: Pollinators' visits in artificial flowers. (a) Artificial iris flower being visited by a male *Eucera* bee. (Photo credit: Y. Sapir). (b) Total number of male *Eucera* bees observed resting in artificial iris flowers in six size categories for all bees in the experiment (16 nights, *N* total visits = 101). Different letters show significant differences based on Tukey tests ($P < 0.001$).

genotype level, showed mostly positive values (Supplementary Fig. S10), indicating that the flower tunnel always warms up faster than the outside, just in variable rates. On average in NET and KUR populations, the flower tunnel warms up to 18 °C ~10 min faster than outside the flower, while in YER the flower tunnel warms up ~35 min faster (Fig. 3e). Within populations, no associations between the flower's warming rate and flower size or black patch size were found (Supplementary Fig. S8b, c).

Fitness and selection analysis

In *I. atropurpurea* populations, the number of flowers per genotype did not differ between NET (15.5 ± 2.5) and KUR (15.5 ± 2.2), as well as number of fruits per genotype (NET: 4.9 ± 1.1, KUR: 3.8 ± 0.5); however, fruit set was higher in KUR than NET (35% vs. 26%, respectively, Supplementary Table S2). In the *I. petrana* Yeruham population, the number of flowers per genotype, fruit and seed set could not be calculated due to the low number of individuals that flowered in the 2021 season (see above). Flower size did not differ significantly in Yeruham between 2017 and 2021 (Supplementary Fig. S6a), and therefore, we used data from 2017 for the number of seeds per fruit. Unfortunately, the number of flowers/fruits per genotype was not available for the 2017 season; thus, total fitness per genotype could not be estimated. Mean number of seeds per fruit differed significantly between populations (LM, $F_{2,137} = 12.64$, $P < 0.001$). Mean number of seeds per fruit in *I. atropurpurea* populations significantly differed, with a lower number in the large-size population NET (11.4 ± 1.8) compared to the medium-size population KUR (28.6 ± 3.2, Tukey test $P < 0.01$); the latter did not differ significantly from *I. petrana* population YER (25.2 ± 2.1, Tukey test $P > 0.05$, Fig. 4a).

Relative fitness based on the number of seeds per genotype did not depend on flower size, nor black patch size in *I. atropurpurea* (all $P > 0.05$). Total number of flowers per genotype had an effect on the relative fitness in the NET population only ($P = 0.04$). We found no selection in any population on flower size in either of *I. atropurpurea* populations (NET: $\beta = -0.52$, $P = 0.325$; KUR: $\beta = -0.15$, $P = 0.433$, Supplementary Fig. S11). We also did not find selection on black patch size (NET: $\beta = 0.52$, $P = 0.632$; KUR: $\beta = -0.06$, $P = 0.720$) or number of flowers (NET: $\beta = 0.02$, $P = 0.07$; KUR: $\beta = -0.005$, $P = 0.535$; Table 1). In the *I. petrana* YER population, we found significant positive directional selection on flower size ($\beta = 0.14$, $P = 0.041$), but not on black patch size ($\beta = 0.10$, $P = 0.112$; Table 1; Fig. 4b).

Experiment 3: Flower heating mechanism and the role of the black patch

Black patch anatomy

The black patch located on the lower tepals at the entrance of the tunnel (i.e. shelter) has conical epidermal cells (Fig. 5a, d) and a very high pigment concentration (C.J. van der Kooi, personal observation). Petal thickness increases from the tip to the black patch and the base of the tunnel (Fig. 5b–e), which is observed in other flowers (e.g. van der Kooi and Stavenga 2019).

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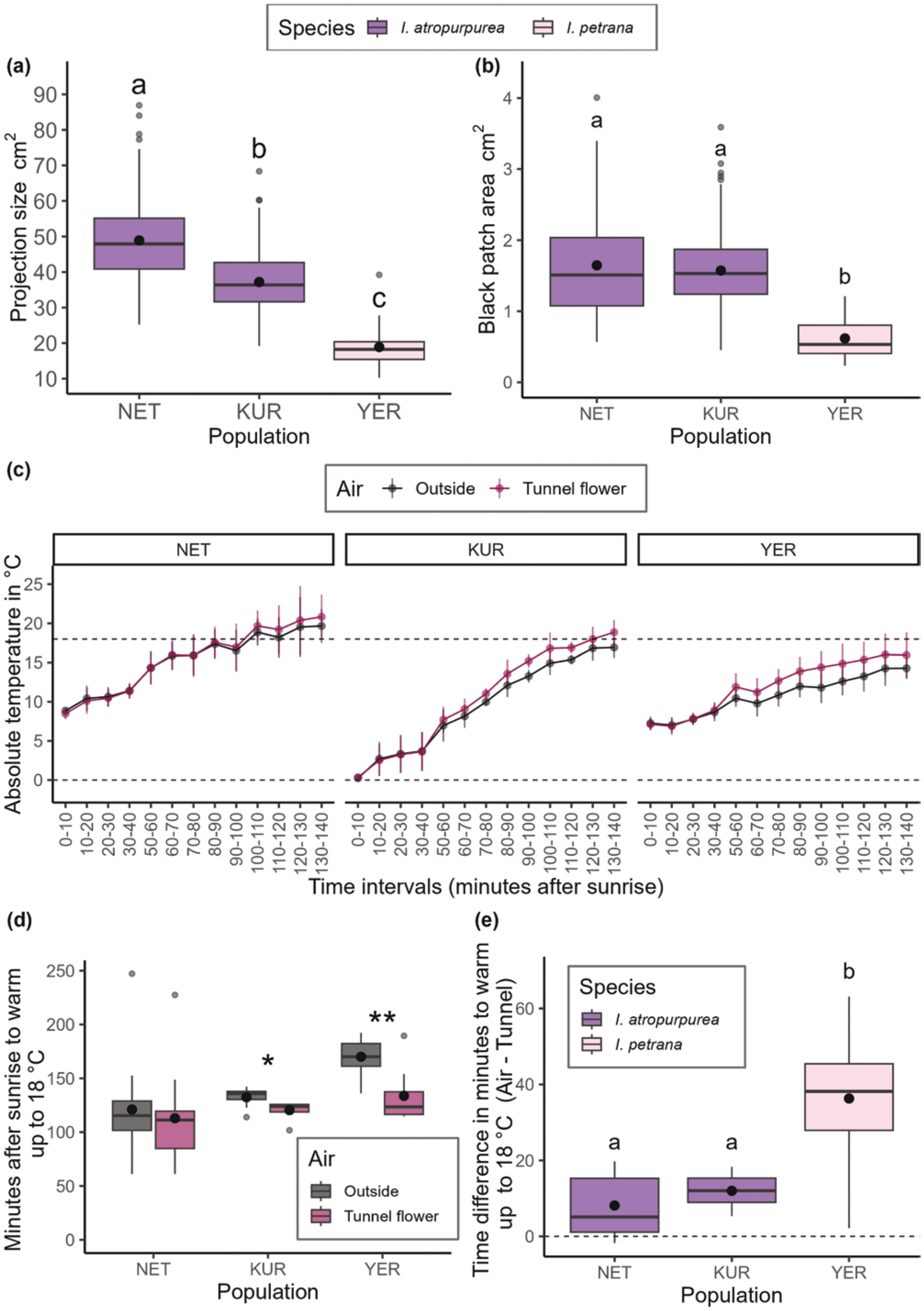

Figure 3: Flower size, black patch size and flower temperature per population. (a) Flower size decreased from NET to KUR and YER populations located from north to south, while (b) black patch size varied between species only. (c) Temperature (mean ± SE) increases with time inside the flower tunnel compared to ambient air temperature. Dashed line denotes 18 °C, the minimum temperature for *Eucera* bees to fly and forage (Stone and Willmer 1989). (d) Time (minutes) from sunrise to warm up to 18 °C in the flower tunnel and ambient air. Boxes describe the time after sunrise to warm up to 18 °C per individual within each population; significance: $*P < 0.05$, $**P < 0.01$. Time difference in (e) refers to the difference in minutes that the air outside-to-inside the tunnel flower takes to get up to 18 °C. Flower tunnel in NET and KUR warms up ~10 min faster than outside air, while in YER ~35 min faster.

Black patch manipulations

We compared the time difference in minutes that the air outside-to-inside the tunnel flower take to get up to 18 °C (see methods) of untouched "Control" flowers to flowers with three manipulations, without the cover of the tunnel ("No ceiling"), with covered

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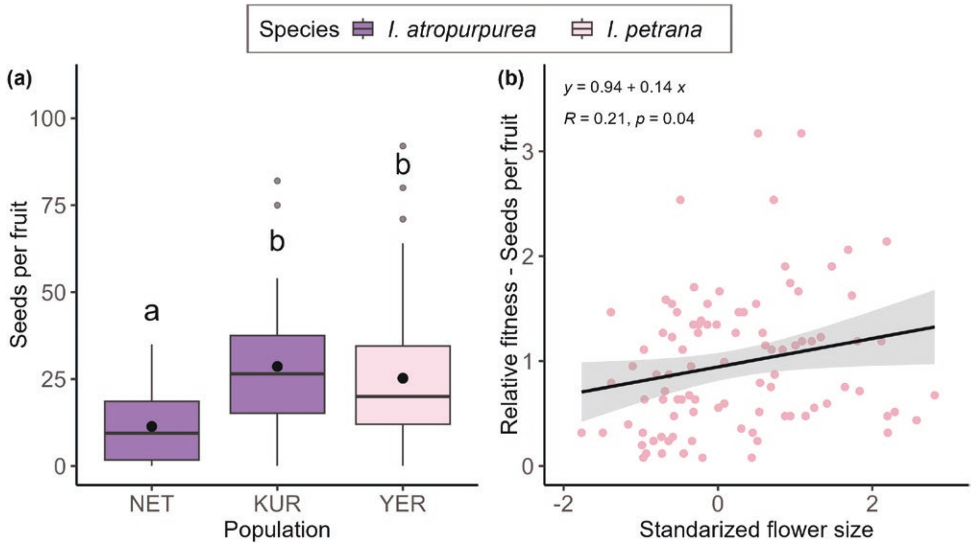

Figure 4: Fitness and selection in YER population. (a) Fitness, measured as seeds per fruit per genotype, in three *Iris* populations. Boxplots show the variation of fitness per individual within each population. (b) Positive selection for flower size was found in *Iris petrana* YER population only. following Lande and Arnold (1983). Due to low flowering in YER populations, seeds were measured in 2017; NET and KUR populations did not show any selection (Table 1, Figure S10). Different letters above boxplots represent significant differences after Tukey post-hoc comparisons ($P < 0.001$). Black dots inside the box plots show mean values. R = Pearson correlation coefficient.

Table 1: Selection analyses on flower and black patch sizes.

	NET ($n = 37$)	KUR ($n = 46$)	YER ($n = 122$)
Trait	**β ± SE**	**β ± SE**	**β ± SE**
Flower size	0.52 ± 0.52	−0.15 ± 0.19	**0.15 ± 0.08***
Black patch size	−0.25 ± 0.52	−0.06 ± 0.18	0.10 ± 0.06
Number of flowers	0.02 ± 0.01	−0.005 ± 0.009	—

Relative fitness was calculated based on seeds per genotype, and linear selection gradients (β ± SE) were calculated on flower size, black patch size and number of flowers per genotype in *Iris atropurpurea* populations (NET and KUR) and not including the number of flowers in *Iris petrana* YER population. *$P < 0.05$, **$P < 0.01$.

black patch ("Patch covered"), and with only the black patch ("Only patch"). The time difference did not vary among manipulations, except for the treatment ‘No ceiling’ with significantly lower time difference ($P < 0.05$), indicating no difference in time to warm up to 18 °C between ambient air and the flower tunnel. Manipulated flowers where the black patch was left isolated or covered did not show any difference compared to control flowers (Fig. 5f).

DISCUSSION

Establishing the link between floral display traits and rewards is essential for understanding how pollinators interact with plants and their impact on plant fitness. We investigated the association between flower size, black patch size, flower temperature and female fitness in populations of nectarless, self-incompatible Royal irises in Mediterranean and arid climatic regions. We hypothesized that flower and black patch sizes are positively associated with flower temperature and that flower and black patch sizes are under positive selection by pollinators. In addition, we expected the black patch to play an important role in flower heating. Our results using artificial flowers indeed showed a pollinator preference for large sizes. However, positive directional selection for larger flowers was found only in YER population with the smallest flowers, and selection for black patch size was not found in any population. Flowers reached

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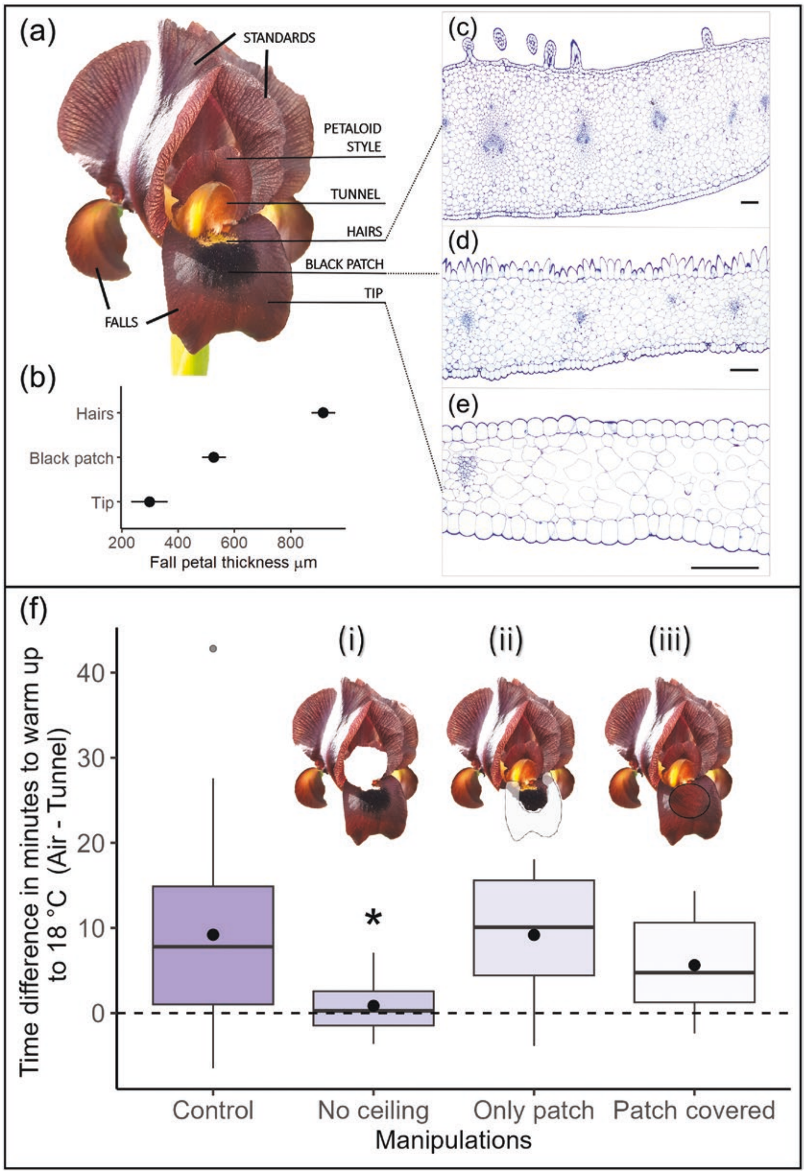

Figure 5: Black patch characteristics and its relationship with flower temperature. (a) Iris flowers have two kinds of tepals: three upper ones called "standards" and three lower ones called "falls". The pollination tunnel, where the bees shelter, is formed by the petaloid style with a stigmatic surface at the tip and another inside; below it, the base part of the fall tepals form a landing platform with yellow hairs, and a black patch is located at the center of the tepal at the entrance of the tunnel. (b) Thickness of the fall petals decreases from hairs to the black patch and even more towards the fall tip. Cross-sections show conical cells in the (d) upper surface of the black patch, (c) flat cells at the hairs and (e) convex cells at the tip. (f) Modified flowers (i) without "ceiling" (petaloid style + anthers), (ii) "only patch" leaving only the hairs and black patch and (iii) "patch covered" whose patch was covered it with a petal of another flower, were compared to untouched control ones to test for flower temperature differences measured as the difference in minutes that the air outside-to-inside the tunnel flower take to get up to 18 °C (take-off minimum temperature of *Eucera* bees). Manipulations were performed on *Iris atropupurea* populations only. Scale bar = 200 μm. Tissue pictures: M. Kraaij, flower: S. Lozada-Gobilard.

18 °C—the minimum temperature for *Eucera* bees to start flying and foraging—faster than the ambient air. On average, flowers from NET and KUR populations warmed up about 10 min faster, and the small-flowered population YER about 35 min faster than the outside air, suggesting that the smaller night shelter in the flower tunnel might provide more favorable conditions for bees to fly and forage sooner than the outside environment. Within populations, this time difference was not related to flower size or black patch size. Finally, we found no evidence to support that the black patch is involved in flower heating.

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Are larger flowers preferred by pollinators?

Pollinators prefer large artificial flowers, but selection for size was evident only in the small-flowered population. We found that night-sheltering bees prefer to sleep in intermediate/large-size flower categories, while there were less visits to the very large flowers. Most bees visit artificial flowers between 76 and 105 cm^2 (Fig. 2b), but this range does not match the most common size in the natural populations, (30–60 cm^2 in *I. atropurpurea* and 15–20 cm^2 in *I. petrana*, Fig. 3a). The largest flowers observed in the NET population were 87 cm^2 corresponding to category 4 (76–95 cm^2), but most of the flowers in NET correspond to size category 2 (40–60 cm^2). Artificial flowers were constructed mainly based on the sizes of *I. atropurpurea*, where size Categories 5 and 6 aimed to expand the sizes observed in nature. Nonetheless, these size categories can be found in other species of Royal irises, such as *I. bismarckiana* and *I. hermona*, growing up to 100–120 cm^2 (Lozada-Gobilard *et al.* 2023; Sapir *et al.* 2002).

Even though the flower size of *I. petrana* is not directly represented by the artificial flowers, the positive selection on flower size in the YER population corroborates the preference of bees for larger flowers. In the other natural populations of *I. atropurpurea*, pollinators may still prefer larger flowers, but selection might not be detected due to the limited variation in flower size within the populations, possibly resulting from a small sample size (Table 1). Another explanation could be that in these populations, flower size might be under balancing selection by environmental stress. Water evaporation can exert negative directional selection on flower size, and the present size distribution is a balance between positive pollinator-mediated selection and water-mediated selection. These two explanations—limited trait variation and balancing selection—are not mutually exclusive and may interact along the aridity gradient. Floral trait plasticity could allow plants to adjust their traits in response to local environmental conditions, whereas context-dependent selection regimes may cause the strength and direction of selection to vary across sites. For example, in drier populations, environmental stress such as water loss may counteract pollinator preferences for larger flowers. In addition, flower size has been identified as a plastic trait in some species in response to pollinators (Harder and Johnson 2005) or water availability (e.g. Lambrecht 2013; Rose-Person *et al.* 2024). Together, these factors can shape the observed patterns of selection on floral traits. More studies across a wider range of populations and conditions are needed to disentangle the relative importance of various agents of selection.

Flower and black patch sizes and their relation to flower temperature

The size of the black patch did not differ between *I. atropurpurea* populations, despite the marked differences in flower size (Fig. 3a, b; Supplementary Fig. S12). These results suggest that the size of the black patch might be under stabilizing selection, independent of the variation of flower size. Indeed, it was recently identified in *I. petrana* that annual differences in mean flower size correlate to mean annual precipitation across years, whereas the black patch size remained constant and independent of precipitation changes (Lozada-Gobilard *et al.* 2022). This again suggests that flower size in irises is a plastic character that might be hardly, or not, under pollinator-mediated selection, contradicting previous results by Lavi and Sapir (2015), who found pollinator-mediated selection explicitly on flower size.

It is important to clarify that whereas flower size and patch size may influence pollinator behavior, we cannot conclude that selection was pollinator-mediated, as fitness was not measured separately in pollen-supplemented and control flowers, as in Lavi and Sapir (2015). Thus, the role of pollinators in driving selection remains uncertain. Still, in the small-flower YER population, positive selection on flower size supports the idea that they may serve as honest signals for larger shelters. However, whether they also signal a heat reward remains unclear due to the limited temperature data collected within the population and a relatively small sample size. Future research should explore the potential for conflicting selection, where larger flowers indicate larger shelters, yet larger shelters may provide less temperature increase.

No evidence for the contribution of the black patch to flower heating

Morphologically unique characteristics of the black patch (i.e. conical cells, the high pigment concentration) might be involved in the flower heating mechanism. Floral manipulations in the field, however, did not support our hypothesis that the black patch plays a role in warming of the flower. In our manipulation experiments, the warming rate measured as time difference (Air—Tunnel) with "only patch" and "patch covered" did not differ from

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untouched control flowers (Fig. 5f), suggesting that the presence of the black patch does not improve the warming rate of the flowers. On the other hand, it is known that conical epidermal cells create a spatially homogenous (matte) visual signal (Stavenga *et al.* 2020; van der Kooi *et al.* 2014). The spatially consistent visual appearance, together with the extremely dark patch, may make the flowers easier for pollinators to detect (Stavenga *et al.* 2020). We found no evidence that supports the role of the black patch in heating the flower, but it might be an important cue for pollinators guiding them towards the entrance of the shelter.

Finally, it is important to note that the third experiment investigating the role of the black patch in floral warming is limited by relatively small sample sizes for the manipulated treatments ($n = 9–10$ per group). We acknowledge that the low sample size may reduce the statistical power to detect subtle effects. Therefore, the absence of a detected effect of the black patch on warming rate should be interpreted with caution. Future studies are needed to more robustly assess the potential thermoregulatory role of the black patch.

Do flower shelters warm up enough to be considered as a reward for the bees?

The temperature reported here corresponds to the air cavity of the flower tunnel, but its surface or top temperature may also play a role, as it could transfer heat to bees through direct contact. Since the flower tunnel surface and top temperatures are not significantly different from the air cavity temperature (Supplementary Fig. S13), the latter provides a reasonable approximation of the temperature experienced by the bees. This suggests that measuring air cavity temperature is a reliable way to assess the thermal environment bees encounter inside the flower and that this heat could serve as a potential floral reward for visiting bees.

The minimum temperature for *Eucera* bees to start flying and foraging is 18 °C (Stone and Willmer 1989). Assuming that the bee's body temperature is similar to the ambient temperature (Heinrich and Esch 1994), the temperature inside the flower tunnel determines when the bees are ready to fly away. If flowers warm up faster than the surrounding air, they could provide a “head start” by accelerating their warming rate. Indeed, we found that the warming inside the flower is faster, suggesting that the flower may give the bees an advantage over those resting outside.

Even though the time to reach 18 °C can be as much as ~35 min faster than ambient air (Fig. 3e), the flower still needs between 80 and 140 min after sunrise to reach this temperature (Fig. 3c, d). This time interval does not match the previously observed bee emergence from these irises, which typically occurs between 60 and 80 min according to Monty *et al.* (2006) and Sapir *et al.* (2006). This mismatch between flower warming and bee emergence may be context-specific, as the minimum air temperature required for foraging varies between species from cooler and warmer climates (Stone and Willmer 1989). In addition, bees may bask in sunlight or increase their metabolic rate to cope with cooler conditions, or delay their activity until the flowers reach optimal temperatures.

Our data shows a slow yet consistent increase in absolute temperature, mainly in NET and KUR populations (Fig. 3c), putatively resulting in a prolonged heat accumulation in these flowers compared to small-sized ones, likely due to greater radiant energy uptake. It is known that during the day, flowers remain warm with a mean temperature difference to the ambient air of 0.5–2 °C (Monty *et al.* 2006; Sapir *et al.* 2006). We hypothesize that heat retention in large flowers might be preferred by the bees at sunset. Follow-up studies should focus on pollinator preferences and temperature changes during the whole day on different flower size ranges, especially on whether pollinators search for long heat heat-accumulating large flowers at sunset.

CONCLUSIONS

We studied the relationship between flower size and flower temperature in the Royal irises using artificial flowers and in three natural populations. Our results suggest that in Royal irises, larger flowers are preferred by the pollinators and might primarily serve as a visual display to attract them. Whereas the black patch might function as a visual cue for pollinators, guiding them to the shelter entrance, rather than absorbing and transferring heat. The temperature increase within the flowers was driven by sunrise radiation in the morning, and it was independent of flower or black patch size. The rapid warming inside flowers might provide a “head start”, likely allowing bees to start foraging sooner than they would after having spent the night outside the flowers. This time advantage may play a key role in pollinator attraction and reproductive success across different climates. Future research should explore whether large flowers

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retain warmth longer, especially into the evening, and examine how flower size influences the behavior and sheltering choices of bees over the course of the day.

Supplementary Material

Supplementary material is available at *Journal of Plant Ecology* online.

Method S1: Methods for creating artificial flowers.

Table S1: Mean ± SEM vertical length, width and projection size of artificial flowers.

Table S2: Number of flowers and individual plants (genotypes) measured and analyzed.

Figure S1: Set up of artificial flowers at the terrace at Tel Aviv University Botanical Garden (TAUBG; 32°06″ N, 34°48″ E), where vegetation represents a Mediterranean habitat and supports a large population of *Eucera* bees and rich community of other wild pollinators.

Figure S2: Methodology used to measure flower size.

Figure S3: Fit of linear regression per flower in NET population for slope calculation.

Figure S4: Fit of linear regression per flower in KUR population for slope calculation.

Figure S5: Fit of linear regression per flower in YER population for slope calculation.

Figure S6: *Iris petrana* flower size, mean annual rainfall and maximum ground temperature between 2017 and 2021.

Figure S7: Size measurements of the entire flower and the black patch.

Figure S8: Relationship between flower, black patch size and time to warm up to 18 °C within populations.

Figure S9: Slopes of absolute temperature inside vs. outside flower tunnel.

Figure S10: Time difference in minutes that the air outside-to-inside the tunnel flower take to get up to 18 °C per genotype.

Figure S11: Selection analyses based on seeds per fruit in *Iris atropurpurea*.

Figure S12: Flower size variation within genotypes.

Figure S13: Absolute temperature inside the flower tunnel at different flower positions: air cavity “tunnel”, surface “floor” and top “ceiling” in comparison to outside ambient air.

Authors' Contributions

Y.S., I.K., M.K.G. and S.L.G. designed the study. O.B., M.K.G., R.H., M.G. and S.L.G. collected the data with the help of Y.B. and Y.S. K.G. and O.B. performed the first experiment. R.H. did the flower manipulations in the field. C.J.v.d.K. and M.K. performed the cross-sections and tissue measurements. S.L.G. analyzed the data and wrote the manuscript. All coauthors contributed significantly with helpful discussions and worked together on the final version of the manuscript.

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Conflict of interest statement. The authors declare that they have no conflict of interest.

Data Availability

Raw data and R script code for this manuscript are provided on Figshare: https://figshare.com/s/59378d3238afb4d5bc77.

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